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<p>The major goal of this research project was to optimize the conjugation procedures used in the preparation of the EGF-Gen conjugates in order to overcome the major limitations of this technology, including (1) instability of the conjugates, (2) presence of Gen-free modified EGF in the conjugate preparations, (3) slow kinetics of cell kill, and (4) low yield of intact conjugate. The research did not lead to a solution for any of the above listed problems/limitations of the technology. It is therefore unlikely that the Genistein conjugates of EGF will be clinically useful. We have explored the potential of EGF conjugates containing other more active inhibitors of the EGF tyrosine kinase. While these conjugates have not been tested in animal models of breast cancer during the term of this grant, they are unlikely to offer significant advantages over the unconjugated small molecule tyrosine kinase inhibitors.</p>				
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## INTRODUCTION

The major goal of this research project was to optimize the conjugation procedures used in the preparation of the EGF-Gen conjugates in order to overcome the major limitations of this technology, including (1) instability of the conjugates, (2) presence of Gen-free modified EGF in the conjugate preparations, (3) slow kinetics of cell kill, and (4) low yield of intact conjugate. Our efforts are summarized in the annual reports which are attached to this report.

## BODY

The research accomplishments associated with each Task outlined in the approved Statement of Work were described in the annual reports, which are included as Documents 1-4 of this submission.

- 1997 REPORT (Document 1)
- 1998 REPORT (Document 2)
- 1999 REPORT (Document 3)
- 2000 REPORT (Document 4)

Our efforts have failed. The research did not lead to a solution for any of the above listed problems/limitations of the technology. It is therefore unlikely that the Genistein conjugates of EGF will be clinically useful. As presented at the last Year 2000 Era of Hope Meeting, we have also explored the potential of EGF conjugates containing other more active inhibitors of the EGF tyrosine kinase. While these conjugates have not been tested in animal models of breast cancer during the term of this grant, they are unlikely to offer significant advantages over the unconjugated small molecule tyrosine kinase inhibitors.

## REPORTABLE OUTCOMES

- **Presentations.** Preliminary studies conducted at the University of Minnesota were reported in Clinical Cancer Research:
  1. **Uckun FM**, Narla R, Zeren T, Yanishevski Y, Myers DE, Waurzyniak B, Ek O, Schneider E, Messinger Y, Chelstrom LM, Gunther R, Evans W. In Vivo Toxicity, Pharmacokinetics, and Anti-Cancer Activity of Genistein Conjugated to Human Epidermal Growth Factor. *Clinical Cancer Research*, 4(5):1125-1134, 1998.
  2. **Uckun FM**, Jun X, Narla RK, Zeren T, Venkatachalam T, Waddick K, Rostosky A, Myers DE. Cytotoxic Activity of EGF-Genistein Against Human Breast Cancer Cells. *Clinical Cancer Research*, 4(4):901-912, 1998.
- The results of work done at the Parker Hughes Institute were presented at the Era of Hope meetings. Manuscripts detailing the negative results with the various EGF-Gen constructs have not yet been completed.
- **Patents and Licenses.** No patentable discoveries resulted from the funded research.

- **Degrees obtained that are supported by this award.** Some of the initial work performed as preliminary studies at the University of Minnesota was included in the Ph.D. thesis of Tamer Zeren, a former graduate student in the Biophysical Sciences Program at the University of Minnesota to fulfill the requirements of the University of Minnesota Graduate School.

- **Informatics**

1. A SCID mouse model of human breast cancer has been established. This model may be used for evaluation of the anti-breast cancer activity of any new anticancer drug.
2. Confocal imaging techniques using multiphoton and single photon confocal laser scanning microscopy have been developed that can be used to evaluate the activity of new anticancer drugs against primary human breast cancer cells. These techniques may prove useful in designing tailored therapies adjusted to the chemosensitivity of cancer cells from each patient.

- **Funding applied for based on work supported by this award**

1. A grant application was submitted to DOD to evaluate the clinical potential of EGF-Gen in a Phase I study since the preclinical studies have not been validated as predictors of clinical outcome. This grant application (BC994075) was not funded.
2. Other grants are being projected that will employ the informatics derived in part from this project (i.e., SCID mouse models and confocal imaging methods).

## **CONCLUSIONS**

Each annual report detailed the conclusions from the experimentation (see Documents 1-4)

## **PERSONNEL**

The following personnel received salary support from this research project:

Fatih M. Uckun (PI)  
Roland Gunther (PI)  
Charles Bedros  
Daiva Kristapaitis  
Elizabeth Lisowski  
Dorothea Myers  
Rama Krishna Narla  
Jack Risdahl  
Deborah Todhunter  
Lisa Tuel-Ahlgren  
Scott Walden  
Kirstin Warner

## **REFERENCES**

Each annual report included a list of all relevant and applicable literature references (see Documents 1-4)

## **APPENDICES**

Attached.

# **APPENDIX**

Document 1: Year 1997 Annual Report

Document 2: Year 1998 Annual Report

Document 3: Year 1999 Annual Report

Document 4: Year 2000 Annual Report

## **DOCUMENT 1. Year 1997 Annual Report**

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## I. INTRODUCTION

Human epidermal growth factor (EGF) is a 53 amino acid, single-chain polypeptide (Mr 6216 daltons), which exerts biologic effects by binding to a specific 170 kDa cell membrane epidermal growth factor receptor (EGF-R/ErbB-1). Many types of cancer cells display enhanced EGF-R expression on their cell surface membranes. Enhanced expression of the EGF-R on cancer cells has been associated with excessive proliferation and metastasis. Examples include breast cancer, prostate cancer, lung cancer, head and neck cancer, bladder cancer, melanoma, and brain tumors. In breast cancer, expression of the EGF-R is a significant and independent indicator for recurrence and poor relapse-free survival. The human EGF-R consists of an extracellular domain with a high cysteine content and N-linked glycosylation, a single transmembrane domain, and a cytoplasmic domain with protein tyrosine kinase (PTK) activity. Binding of EGF to the EGF-R/ErbB-1 results in receptor dimerization with itself or other members of the Erb-B (subtype I) transmembrane PTK family (e.g., Erb-B2, Erb-B3), resulting in activation with autophosphorylation of the PTK domain. EGF-R is physically and functionally associated with Src protooncogene family PTK, including p60<sup>sc</sup>. This association is believed to be an integral part of the signaling events in breast cancer cells mediated by the EGF-R and contributes to proliferation and survival of breast cancer cells.

Our recent studies provided evidence that the membrane-associated EGF-receptor (R)-protein tyrosine kinase (PTK) complexes serve as endogenous negative regulators of apoptosis in breast cancer cells. We therefore postulated that the EGF-R, similar to the CD19 receptor on leukemia and lymphoma cells, may be a suitable target for biotherapy using tyrosine kinase inhibitors. Genistein (Gen), an isoflavone (5,7,4'-trihydroxyisoflavone) from fermentation broth of *Pseudomonas spp.*, is a naturally occurring tyrosine kinase inhibitor present in soybeans. We found that targeting Gen to the EGF-R-PTK complexes in breast cancer cells using the EGF-Gen conjugate triggers apoptotic cell death. The purpose of this research project is to further evaluate the clinical potential of this membrane-directed apoptosis

induction strategy by examining the *in vivo* toxicity profile, pharmacokinetics, and efficacy of EGF-Gen in preclinical animal model systems.

## II. BODY

### II.1. IN VITRO STUDIES

#### A. EXPERIMENTAL METHODS

**Preparation of the EGF-Gen.** rhEGF was produced in *E. coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies and the mature protein was recovered by trypsin-cleavage followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of  $4.6 \pm 0.2$ . The endotoxin level was 0.172 EU/mg. The recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) (18) has been employed in the synthesis of the EGF-Gen conjugate. Sulfo-SANPAH modified rhEGF was mixed with a 10:1 molar ratio of Gen (LC Laboratories, Woburn, MA) [50 mM solution in dimethyl sulfoxide (DMSO)] and then irradiated with gentle mixing for 10 min with UV light at wavelengths 254-366 nm with a multiband UV light-emitter (Model UVGL-15 Mineralight; UVP, San Gabriel, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a 10-fold molar excess of differentially hydroxyl-protected Gen resulted in the attachment of Gen via its available C7-hydroxyl group to lysine 28 or lysine 48 residues of EGF. Excess Gen in the reaction mixture was removed by passage through a PD-10 column, and 12 kDa EGF-EGF homoconjugates with or without conjugated Gen as well as higher molecular weight reaction products were removed by size-exclusion high-performance liquid chromatography (HPLC). Reverse phase HPLC using a Hewlett-Packard (HP) 1100 series HPLC instrument was used for separation of EGF-Gen from EGF-SANPAH. After the final purification, analytical HPLC was performed using a Spherisorb ODS-2

reverse phase column (250x4 mm, Hewlett-Packard, Cat.# 79992402-584). Prior to the HPLC runs, a Beckman DU 7400 spectrophotometer was used to generate a UV spectrum for each of the samples to ascertain the  $\lambda_{max}$  for EGF-Gen, EGF-SANPAH, and unmodified EGF. Each HPLC chromatogram was subsequently run at wavelengths of 214, 265, and 480 nm using the multiple wavelength detector option supplied with the instrument to ensure optimal detection of the individual peaks in the chromatogram. Analysis was achieved using a gradient flow consisting of 0% to 100% eluent in a time interval of 0 to 30 min. Five  $\mu$ L samples applied to the above column were run using the following gradient program: 0-5 min: 0-20% eluent; 5-20 min: 20-100% eluent; 25-30 min: 100% eluent; and 30-35 min: 100-0% eluent. The eluent was a mixture of 80% acetonitrile (CH<sub>3</sub>CN), 20% H<sub>2</sub>O and 0.1% TFA.

Electrospray ionization mass spectrometry (20, 21) was performed using a PE SCIEX API triple quadrupole mass spectrometer (Norwalk, CT) to determine the stoichiometry of Gen and EGF in EGF-Gen. <sup>125</sup>I-Gen was also used to confirm the stoichiometry of Gen and EGF in EGF-Gen and to verify the removal of free genistein and genistein-labeled EGF-EGF homoconjugates by the described purification procedure. Gen (in 65% ethanol, 35% phosphate buffered saline [PBS], pH 7.5) (LC Laboratories, Woburn, MA) was radioiodinated at room temperature in Reacti-Vials containing Iodo-beads (Pierce Chemical Co., Rockford, IL) and <sup>125</sup>I (Na, carrier-free, 17.4 Ci/mg, NEN, Boston, MA) as per manufacturer's instructions (18, 19). The purity of EGF-<sup>125</sup>I-Gen was assessed by SDS-PAGE (20 % separating gels, nonreducing conditions) and autoradiography using intensifying screens and Kodak XAR-5 film. EGF-<sup>125</sup>I-Gen was also used for in vitro ligand binding assays (18, 21) and EGF-Gen internalization studies (18).

**Breast Cancer Cells.** MDA-MB-231 (ATCC HTB-26) is an EGF-R positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51 year old patient. BT-20 (ATCC HTB-19) is another EGF-R positive breast cancer cell line isolated from the primary breast tumor of a 74 year old patient with grade II mammary adenocarcinoma. MDA-MB-231 and BT-20 breast cancer cell lines were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum. For subculturing, medium was removed from the flasks containing a confluent layer of cells, and fresh 0.25% trypsin was added for 1-2

min. Trypsin was removed and cultures incubated for 5-10 min at 37°C until cells detached. Fresh medium was then added, cells aspirated and dispensed into new flasks.

**Binding of EGF-<sup>125</sup>I-Gen to Breast Cancer Cells.** Ligand binding assays using EGF-<sup>125</sup>I-Gen ( $2.0 \times 10^8$  cpm/ $\mu$ mol), <sup>125</sup>I-Gen ( $3.8 \times 10^8$  cpm/ $\mu$ mol) and <sup>125</sup>I-EGF ( $2.2 \times 10^{12}$  cpm/ $\mu$ mol; Amersham) were performed using standard procedures, as previously described (18, 22). The cell lines in ligand binding assays included the EGF-R positive breast cancer cell lines, MDA-MB-231 and BT-20, as well as the EGF-R negative human leukemia cells lines, NALM-6 (pre-B leukemia) and HL-60 (promyelocytic leukemia).

**Immunocytochemistry.** Immunocytochemistry was used to (i) examine the surface expression of EGF-R on breast cancer cells, (ii) evaluate the uptake of EGF-Gen by breast cancer cells and (iii) examine the morphologic features of EGF-Gen treated cancer cells. In uptake studies, the culture medium was replaced with fresh medium containing 10  $\mu$ g/ml EGF or EGF-Gen and cells were incubated at 37°C for 5 min, 10 min, 15 min, 30 min, 60 min, and 24 hours. For EGF-R expression studies, cells were plated on poly-L-lysine coated glass-bottom 35 mm Petri dishes and maintained for 48 hr. At the end of the incubation, cells were washed with PBS and fixed in 2% paraformaldehyde. The cells were permeabilized and non-specific binding sites were blocked with 2.5% BSA in PBS containing 0.1% Triton X-100 for 30 min. To detect the EGF-R/EGF-Gen complexes, cells were incubated with a mixture of a monoclonal antibody (1:10 dilution in PBS containing BSA and Triton X-100) directed to the extracellular domain of the human EGF-R (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) and a polyclonal rabbit anti-Gen antibody (1:500 dilution) for 1 hr at room temperature. After rinsing with PBS, cells were incubated for 1 hr with a mixture of a goat anti-mouse IgG antibody conjugated to FITC (Amersham Corp., Arlington Heights, IL) at a dilution of 1:40 in PBS and donkey anti-rabbit IgG conjugated to Texas Red (Amersham Corp.). Cells were washed in PBS and counterstained with toto-3 (Molecular Probes Inc., Eugene, OR) for 10 min at a dilution of 1:1000. Cells were washed again with PBS and the coverslips were mounted with Vectashield (Vector Labs, Burlingame, CA) and viewed with

a confocal microscope (Bio-Rad MRC 1024) mounted in a Nikon Labophot upright microscope. Digital images were saved on a Jaz disk and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**In Vitro Treatment of Cells with EGF-Genistein.** In order to determine the cytotoxic activity of EGF-Gen against breast cancer cells, cells in alpha-MEM supplemented with 10%(v/v) fetal calf serum were treated with various concentrations of EGF-Gen for 24 hours at 37°C, washed twice in alpha-MEM, and then used in either apoptosis assays or clonogenic assays, as described hereinafter. Controls included (a) cells treated with G-CSF-Gen (an irrelevant cytokine-Gen conjugate which does not react with EGF-R), (b) cells treated with unconjugated EGF plus unconjugated Gen, (c) cells treated with unconjugated Gen or unconjugated EGF, and (d) cells treated with PBS, pH 7.4. In some experiments, excess G-CSF or EGF were added to the EGF-Gen containing treatment medium to show that the cytotoxicity of EGF-Gen can be selectively blocked by excess EGF but not G-CSF.

**Immune-Complex Kinase Assays and Anti-Phosphotyrosine Immunoblotting.** Twenty-four hours after treatment with EGF-Gen, cells were stimulated with 20 ng/mL EGF for 5 min, lysed in 1% Nonidet P-40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala<sup>351</sup>-Asp<sup>364</sup> of the human EGF-R (Upstate Biotechnology Inc. [UBI] Catalog # 05-104). EGF-R immune complexes were examined for tyrosine phosphorylation by Western blot analysis, as previously described (23). All anti-phosphotyrosine Western blots were subjected to densitometric scanning using the automated AMBIS system (Automated Microbiology System, Inc., San Diego, CA) and for each time point a % inhibition value was determined by comparing the density ratios of the tyrosine phosphorylated EGF-R protein bands to those of the baseline sample and using the formula: % Inhibition = 100 - 100x [Density of tyrosine phosphorylated EGF-R band]<sub>test sample</sub> : [Density of tyrosine phosphorylated EGF-R band]<sub>baseline control sample</sub>. The IC50 values were determined using an Inplot program (Graphpad Software, Inc., San Diego, CA). The Src immune complexes were then subjected to immune complex kinase assays, as described (18, 19, 23).

**Apoptosis Assays.** Loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (24). Plasma membrane permeability to propidium iodide (PI, Sigma) develops at a later stage of apoptosis (24). MC540 binding and PI permeability were simultaneously measured in breast cancer cells 24 hours after exposure to EGF-Gen (either without any cytokine preincubation or following preincubation with excess unconjugated EGF or G-CSF), unconjugated Gen, unconjugated EGF + unconjugated Gen, or G-CSF-Gen, as described (24). Stock solutions of MC540 and PI, each at 1 mg/mL, were passed through a 0.22 µm filter and stored at 4°C in the dark. Shortly before analysis, suspensions containing  $1 \times 10^6$  cells were suspended in 5 µg/mL MC540 and 10 µg/mL PI and kept in the dark at 4°C. Whole cells were analyzed with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). All analyses were done using 488 nm excitation from an argon laser. MC540 and PI emissions were split with a 600 nm short pass dichroic mirror and a 575 nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission and a 635 nm band pass filter was used for PI emission. To detect the DNA fragmentation in apoptotic cells, cells were harvested 24 hours after treatment with EGF-Gen and DNA was prepared from Triton-X-100 detergent lysates for analysis of fragmentation, as described (24). In brief, cells were lysed in hypotonic 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 0.2% Triton-X-100, and subsequently centrifuged at 11,000 g. This protocol allows the recovery of fragmented DNA in the supernatant. To detect apoptosis-associated DNA fragmentation, supernatants were electrophoresed on a 1.2% agarose gel, and the DNA fragments were visualized by ultraviolet light after staining with ethidium bromide.

**Clonogenic Assays.** After treatment with EGF-Gen, G-CSF-Gen, unconjugated EGF, unconjugated Gen, or PBS, cells were resuspended in clonogenic medium consisting of alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/mL/dish and cultured in a humidified 5% CO<sub>2</sub> incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as % inhibition of clonogenic cells at a particular concentration of the test agent using the formula  
$$\text{Inhibition} = (1 - \text{Mean } \# \text{ of colonies [Test]} / \text{Mean } \# \text{ of colonies [Control]}) \times 100.$$
 Furthermore, the

dose survival curves were constructed using the percent control survival (= Mean # of colonies[Test] / Mean # colonies [Control] x 100) results for each drug concentration as the data points and the IC<sub>50</sub> values were calculated. The IC<sub>50</sub> values were determined using an Prism Version II Inplot program (Graphpad Software, Inc., San Diego, CA). The mean IC<sub>50</sub> values for EGF-Gen and Gen were compared using Student's t-tests.

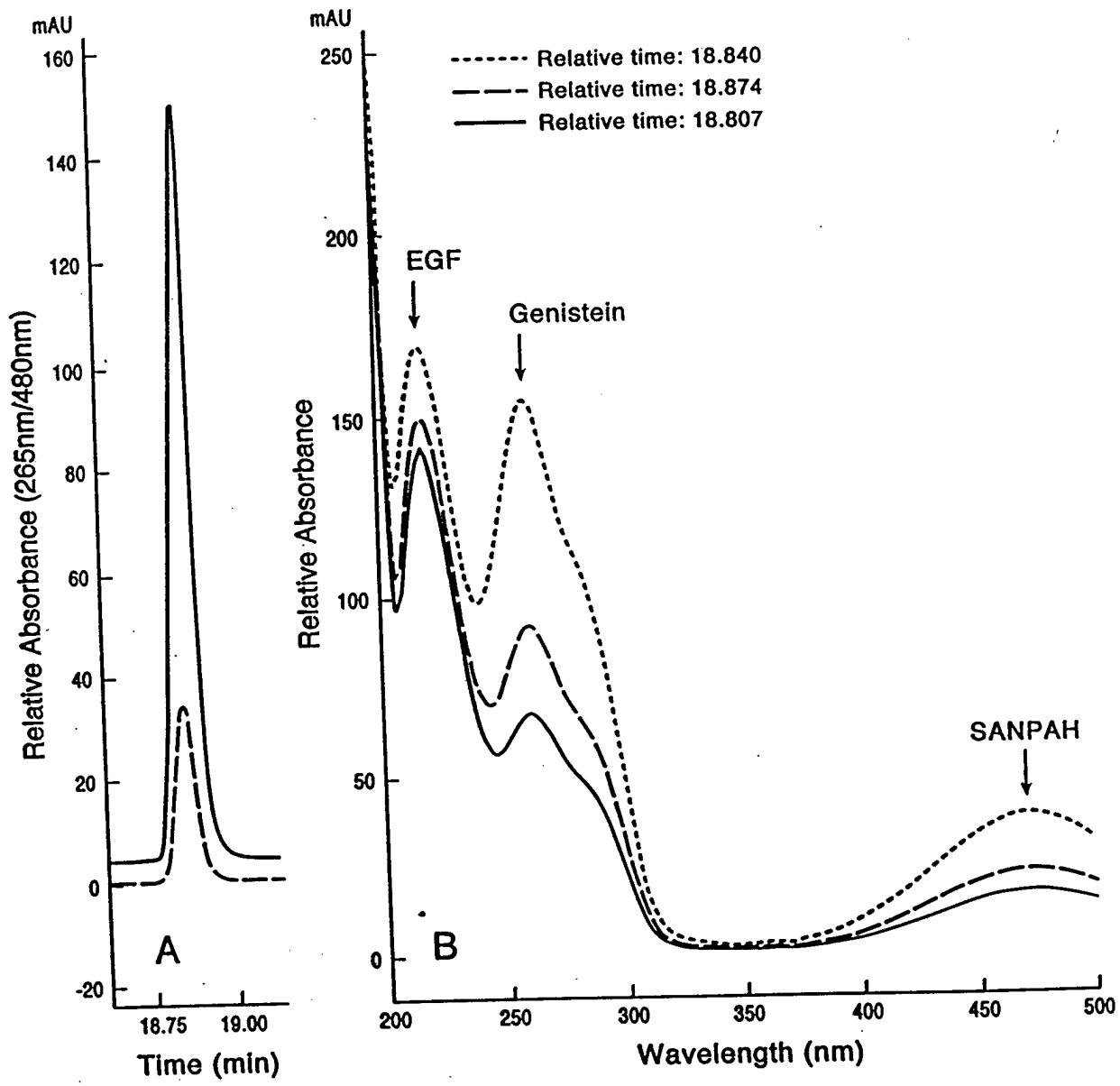
## B. RESULTS

**Composition of EGF-Gen conjugate.** EGF-Gen was consistently found to contain, in four independent conjugations, one molecule of Gen per each EGF molecule, as determined by the specific activity of EGF-Gen prepared with <sup>125</sup>I-Genistein. The electrospray ionization mass spectrum of EGF-Gen also showed a single 7287 kDa EGF-Gen species containing one EGF molecule, three SANPAH molecules, and one Gen molecule. Figure 1A depicts the analytical HPLC chromatogram of purified EGF-Gen, which eluted as a single peak at 18.84 min. The UV spectral scan of this HPLC peak revealed (i) a peak at a wavelength of 220 nm (due to peptide bonds) and a shoulder at 280 nm (due to aromatic amino acid residues) representing EGF, (ii) a peak at 267 nm representing Gen, and (iii) a peak at 480 nm corresponding to the nitrobenzene substituted structure in the SANPAH moiety (Figure 1B). The EGF-Gen conjugate was highly stable in mouse, monkey, and human plasma with no detectable decrease in concentration, as examined by quantitative autoradiography of EGF-<sup>125</sup>I-Gen, as well as quantitative anti-Gen Western blot analysis of non-radioactive EGF-Gen even after 3 days of continuous incubation at 37°C (data not shown).

**Binding of EGF-Genistein to EGF-R-positive Breast Cancer Cells.** We examined the *in vitro* binding of radioiodinated EGF-Gen (EGF-<sup>125</sup>I-Gen, Final concentration: 260 nM = 1700 ng/ml) to EGF-R on these breast cancer cells in the presence and absence of 100-fold molar excess non-radioactive EGF using standard ligand binding assays (18, 19, 22). EGF-<sup>125</sup>I-Gen was able to bind to MDA-MB-231 and BT-20 human breast cancer cells and this binding was blocked by excess nonradioactive EGF (% EGF-Inhibitable Binding = 56% for MDA-MB-231 and 65% for BT-20; 4.5 x 10<sup>6</sup> EGF-Gen molecules/cell for MDA-MB-231 cells and 5.7 x 10<sup>6</sup> EGF-Gen molecules/cell for BT-20 cells; Table 1), but not by excess nonradioactive GM-CSF, which was used as a control ligand (data not shown). EGF-<sup>125</sup>I-Gen did not

## **FIGURE 1**

**(A). HPLC Chromatogram of EGF-Gen.** A sample of the EGF-Gen conjugate was analyzed on a Spherisorb ODS, 250x4 mm reverse-phase column using a 0.1% TFA-H<sub>2</sub>O<sub>2</sub>/0.1% TFA-80% acetonitrile-20% H<sub>2</sub>O<sub>2</sub> gradient as described in the Methods. The retention time of EGF-Gen was 18.84 min. The solid line represents the absorbance at 265 nm and the dotted line represents the absorbance at 480 nm. **(B). UV Spectrum of the EGF-Gen.** The EGF-Gen peak obtained from the HPLC run shown in (B) was further analyzed by the diode array multiple wavelength detector. (see next page)



**FIGURE 1**

bind to EGF-R negative HL60 or NALM-6 leukemia cell lines. EGF-Gen was as effective as unconjugated EGF in blocking the binding of  $^{125}\text{I}$ -EGF to breast cancer cells, whereas GM-CSF did not block the binding of  $^{125}\text{I}$ -EGF (Table 1). Thus, EGF-Gen was able to bind to EGF-R positive breast cancer cells via its EGF moiety. However, since (1) 35-44% of the EGF-Gen binding to breast cancer cells was not inhibitable by excess unconjugated EGF, (2) EGF-Gen binding not inhibitable by excess EGF was also observed with EGF-R negative leukemia cell lines NALM-6 and HL-60, and (3) unconjugated Gen showed binding to all cell lines, which was not inhibitable by EGF, the Gen moiety as well as non-specific surface adherence may also contribute to the observed binding of EGF-Gen to breast cancer cells.

We next examined the kinetics of uptake and cytotoxicity of unlabeled EGF-Gen in BT-20 (Figure 2) and MDA-MB-231 (Figure 3) human breast cancer cells using immunocytochemistry and confocal laser microscopy for tracing the internalized EGF-R and EGF-Gen molecules as well as evaluating the morphologic changes in treated cells. EGF-Gen was very similar to unconjugated EGF with respect to its ability to bind to and induce internalization of EGF-R molecules. Within 5 min after exposure to EGF-Gen, the EGF-R/EGF-Gen complexes begin being internalized, as determined by co-localization of EGF-R (detected by anti-EGF-R antibody, green fluorescence) and EGF-Gen (detected by anti-Gen antibody, red fluorescence) in the cytoplasm of treated cells (Figure 2 & Figure 3). By 15-30 min, the EGF-R/EGF-Gen complexes were detected in the perinuclear region of the cells. The examination of the morphologic features of EGF-Gen-treated (but not EGF-treated) cells after 24 hours of exposure showed distinct changes consistent with apoptosis including marked shrinkage, nuclear fragmentation, and formation of apoptotic bodies (Figure 2).

**Biologic Activity of EGF-Gen.** EGF-Gen treatment resulted in decreased tyrosine phosphorylation of the EGF-R in a dose-dependent fashion (Figure 4A). Whereas EGF-Gen exhibited marked PTK-inhibitory activity in MDR-MB-231 cells at concentrations as low as 0.1  $\mu\text{M}$  in the treatment medium, unconjugated Gen did not significantly affect the EGF-R tyrosine phosphorylation even at a 10  $\mu\text{M}$  concentration (Figure 4A). The inhibitory effect of EGF-Gen was blocked by preincubation of cells

**Table 1. Specific binding of EGF-<sup>125</sup>I-Gen to Breast Cancer Cells**

Cell line	<i>EGF-<sup>125</sup>I-Gen Binding to Breast Cancer Cells</i>						Molecules per cell
	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding	Inhibitable binding	p mol/10 <sup>6</sup> cells		
MDA-MB-231	4531	1983	2548	56%	7.5	4.5x10 <sub>6</sub>	
BT-20	7511	2663	4848	65%	9.5	5.7x10 <sub>6</sub>	
NALM-6	2708	3091	0	None	None	None	
HL-60	788	1346	0	None	None	None	

Cell line	<i><sup>125</sup>I-EGF Binding to Breast Cancer Cells</i>							
	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding (cpm)	Inhibitable binding	+cold EGF-Gen (cpm)	Inhibition by EGF-Gen	+cold GM-CSF (cpm)	Inhibition by GM-CSF
MDA-MB-231	15,102	1,100	14,002	93%	1,398	91%	15,440	0%
BT-20	17,351	ND	ND	ND	1,624	91%	16,486	5%

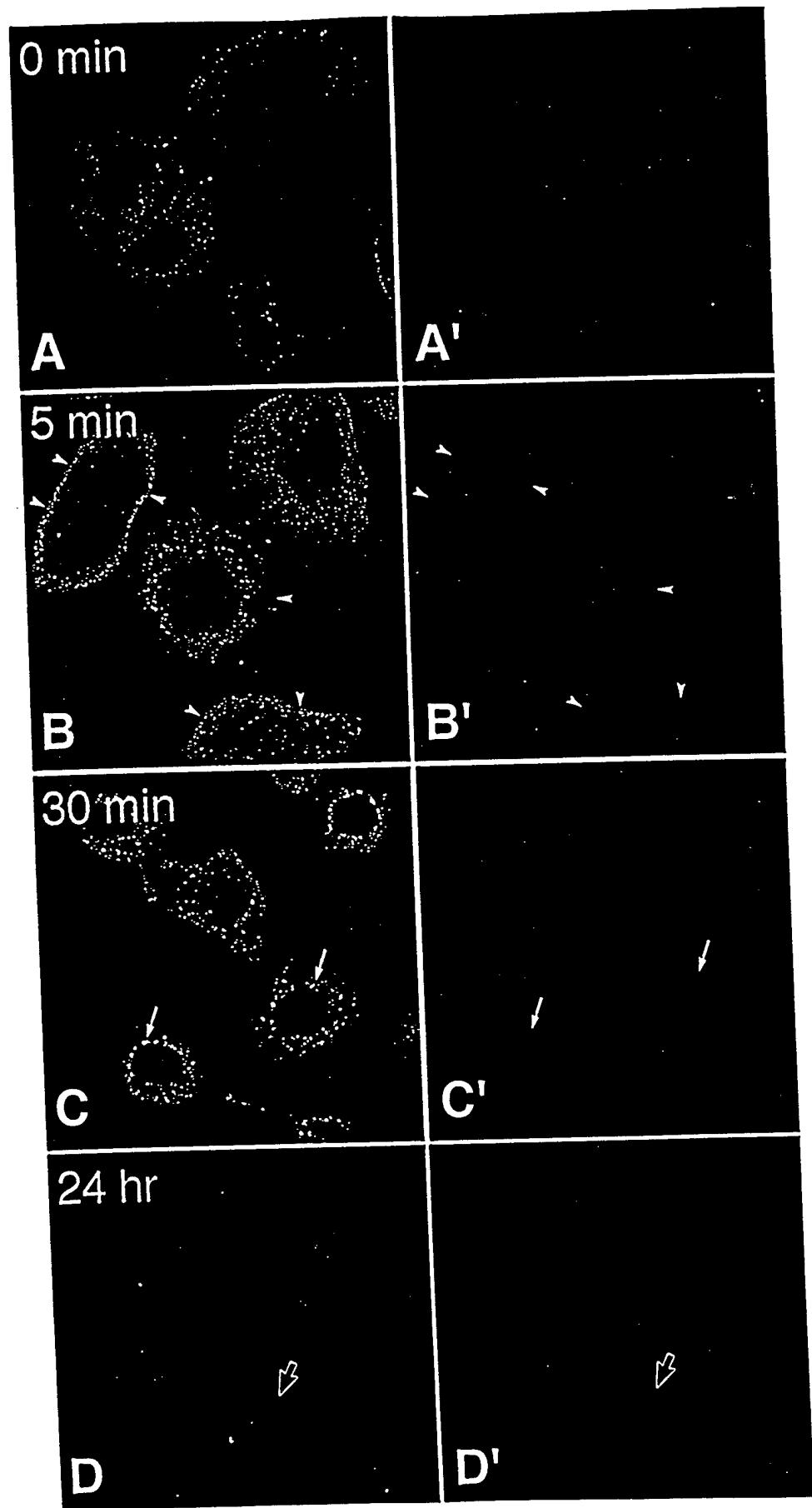
Cell line	<i><sup>125</sup>I-Gen Binding to Breast Cancer Cells</i>						Molecules per cell
	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding	Inhibitable binding	p mol/10 <sup>6</sup> cells		
MDA-MB-231	852	860	0	None	None	None	None
BT-20	2439	2540	0	None	None	N.D.	N.D.
NALM-6	1098	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
HL-60	814	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The binding of EGF-Gen, unconjugated Gen, and unconjugated EGF to EGF-R<sup>+</sup> breast cancer cells and EGF-R<sup>-</sup> leukemia cells was examined in ligand binding assays, as described in Materials and Methods. Each cpm determination was performed in duplicate.

## FIGURE 2

**Binding and Internalization of EGF-Gen in BT-20 Cells.** Cells were incubated with EGF-Gen (10 µg/ml) for the indicated times (0 min: A, A'; 5 min: B, B'; 30 min: C, C'; 24 hr: D, D'). Cells were then processed for immunocytochemistry using a monoclonal anti-EGF-R antibody and FITC conjugated goat anti-mouse IgG for EGF-R (green fluorescence, left panel). Gen was detected using a polyclonal anti-Gen antibody and Texas Red conjugated anti-rabbit IgG (red fluorescence, right panel), as described in Materials and Methods. Blue fluorescence represents the nuclei stained with toto-3. A, A': BT-20 cells showed high level EGF-R expression; no red fluorescent staining was observed in untreated cells incubated with the anti-Gen antibody. B, B': Following 5 min exposure, EGF-Gen was bound to the cell surface EGF-R (arrowheads) and the internalization of the EGF-R was detected by cytoplasmic green fluorescent staining, whereas the internalization of EGF-Gen molecules was evident from the red fluorescent staining. C, C': By 30 min, most of the EGF-R/EGF-Gen complexes were internalized and deposited in the perinuclear region (arrows). D, D': Following 24 hr exposure, the cells lost their adherent features and showed morphologic changes consistent with apoptosis (open arrow).

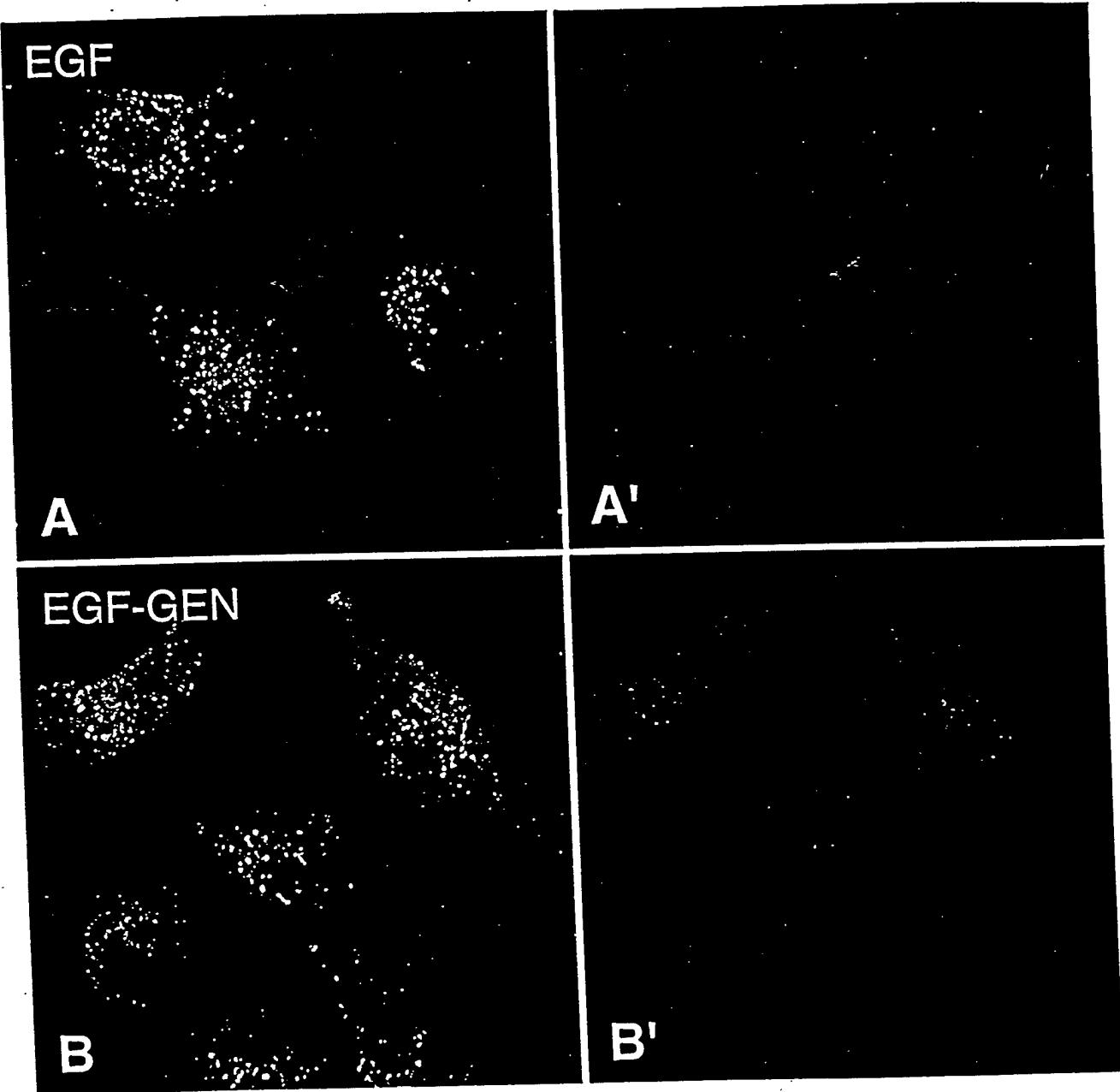
(see next page)



**FIGURE 2**

### **FIGURE 3**

**Binding and Internalization of EGF-Gen in MDA-MB-231 Cells.** Cells were incubated with either unconjugated EGF (10 µg/ml) (A, A') or EGF-Gen (10 µg/ml) (B, B') for 15 min and processed for the detection of EGF-R (green fluorescence; left panel) and EGF-Gen (red fluorescence; right panel) by immunocytochemistry. EGF-Gen was very similar to EGF with respect to its ability to induce internalization of the surface EGF-R molecules. Notably, the intracellular staining patterns for EGF-R and EGF-Gen were very similar. (see next page)



**FIGURE 3**

with excess EGF but not by excess G-CSF, a control cytokine which does not react with EGF-R (Figure 4B). We next used immune complex kinase assays to assess the effects of EGF-Gen on the enzymatic activities of EGF-R associated Src PTK in MBA-MB-231 cells. As shown in Figure 4C, EGF-Gen treatment inhibited the Src kinase. Unlike EGF-Gen, a mixture of unconjugated Gen and EGF or G-CSF-Gen did not inhibit the Src kinase activity in MDA-MB-231 cells. Thus, EGF-Gen is a potent inhibitor of both the EGF-R tyrosine kinase as well as other PTK which are associated with the EGF-R.

Targeting Gen to vital PTK in leukemia cells results in apoptotic cell death (18, 19). Furthermore, the examination of the morphologic features of EGF-Gen treated BT-20 and MDA-MB-231 cells by immunocytochemistry suggested that these cells might be undergoing apoptosis. Therefore, we decided to formally study whether EGF-Gen could trigger apoptosis in breast cancer cells. To this end, we first used a quantitative flow cytometric apoptosis detection assay. MC540 binding and propidium iodide (PI) permeability of MDA-MB-231 breast cancer cells were simultaneously measured before and after treatment with 1 $\mu$ g/ml EGF-Gen ( $=0.1\text{ }\mu\text{M}$ ), 10 $\mu$ g/ml EGF ( $1\text{ }\mu\text{M}$ ) plus 10 $\mu$ g/ml unconjugated Gen ( $=37\text{ }\mu\text{M}$ ), or 1 $\mu$ g/ml G-CSF-Gen. Whereas less than 10% of MDA-MB-231 or BT-20 cells showed apoptotic changes after EGF plus unconjugated Gen treatment or G-CSF-Gen treatment, a significant portion of cells underwent apoptosis within 24 hours after EGF-Gen treatment (95.1% = 57.9% MC540 $^+$  early stage apoptosis plus 37.2% MC540 $^+/\text{PI}^+$  advanced stage apoptosis at 24 hours) (Figure 5). Excess EGF (10  $\mu$ g/ml) but not excess G-CSF (10  $\mu$ g/ml) could prevent EGF-Gen-induced apoptosis. Thus, EGF-Gen causes apoptosis in an EGF-R specific fashion and this activity requires both its EGF-R binding growth factor moiety as well as its PTK inhibitory Gen moiety.

As shown in Figure 6, DNA from Triton-X-100 lysates of EGF-Gen-treated MDA-MB-231 or BT-20 breast cancer cells showed a ladder-like and dose-dependent fragmentation pattern, consistent with apoptosis. The EGF-Gen-induced DNA fragmentation was EGF-R-specific because DNA from cells treated with the control cytokine-Gen conjugate G-CSF-Gen showed no fragmentation. DNA fragmentation was dependent both on the PTK inhibitory function of Gen and the targeting function of

## FIGURE 4

**Inhibitory Activity of EGF-Gen on EGF-R-associated PTKs of Human Breast Cancer Cells.** [A] After a 24-hour incubation with EGF-Gen (0.1  $\mu$ M, 1.0  $\mu$ M, or 10.0  $\mu$ M) or unconjugated Gen (10  $\mu$ M), MDA-MB-231 cells were lysed in 1% Nonidet-P-40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala<sup>351</sup>-Asp<sup>364</sup> of the human EGF-R. The EGF-R immune complexes were then subjected to APT immunoblotting. [B] After a 24-hour incubation with 10.0  $\mu$ g/mL (=1.3  $\mu$ M) EGF-Gen, MDA-MB-231 cells were lysed in 1% Nonidet-P-40 buffer, cell lysates were immunoprecipitated with an anti-EGF-R antibody, and the EGF-R immune complexes were subjected to APT immunoblotting as in [A]. Controls included untreated cells as well as cells pretreated with 10-fold molar excess of unconjugated EGF (=13  $\mu$ M) or unconjugated G-CSF (13  $\mu$ M) prior to EGF-Gen incubation. (C) Src immune complex kinase assays in the presence of [ $\gamma$ -<sup>32</sup>P]ATP (50  $\mu$ Ci/ $\mu$ mol) were performed on the lysates of MDA-MB-231 cells treated with 1  $\mu$ g/mL EGF-Gen (0.1  $\mu$ M), 1  $\mu$ g/mL G-CSF-Gen, or 1  $\mu$ g/mL EGF (0.1  $\mu$ M) + 1  $\mu$ g/mL Gen (3.7  $\mu$ M). Controls included untreated cells. (see next page)

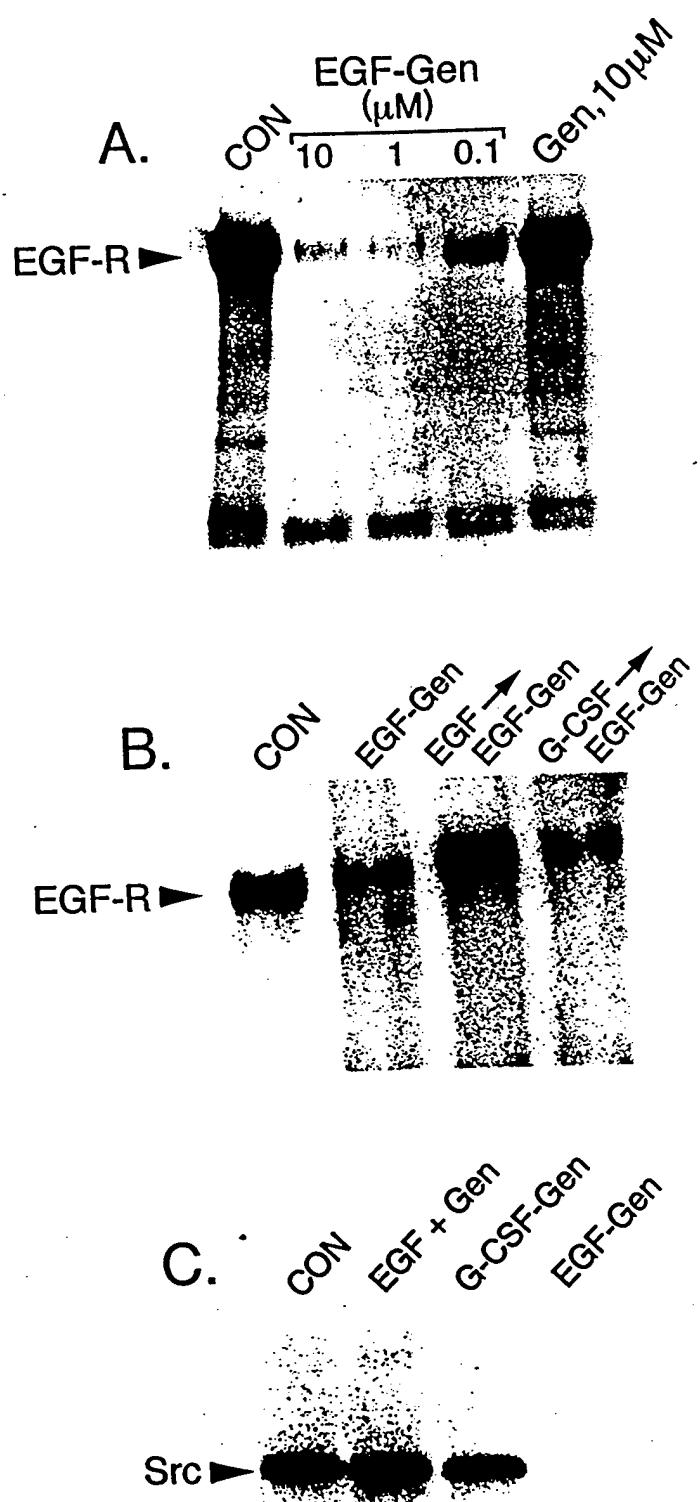
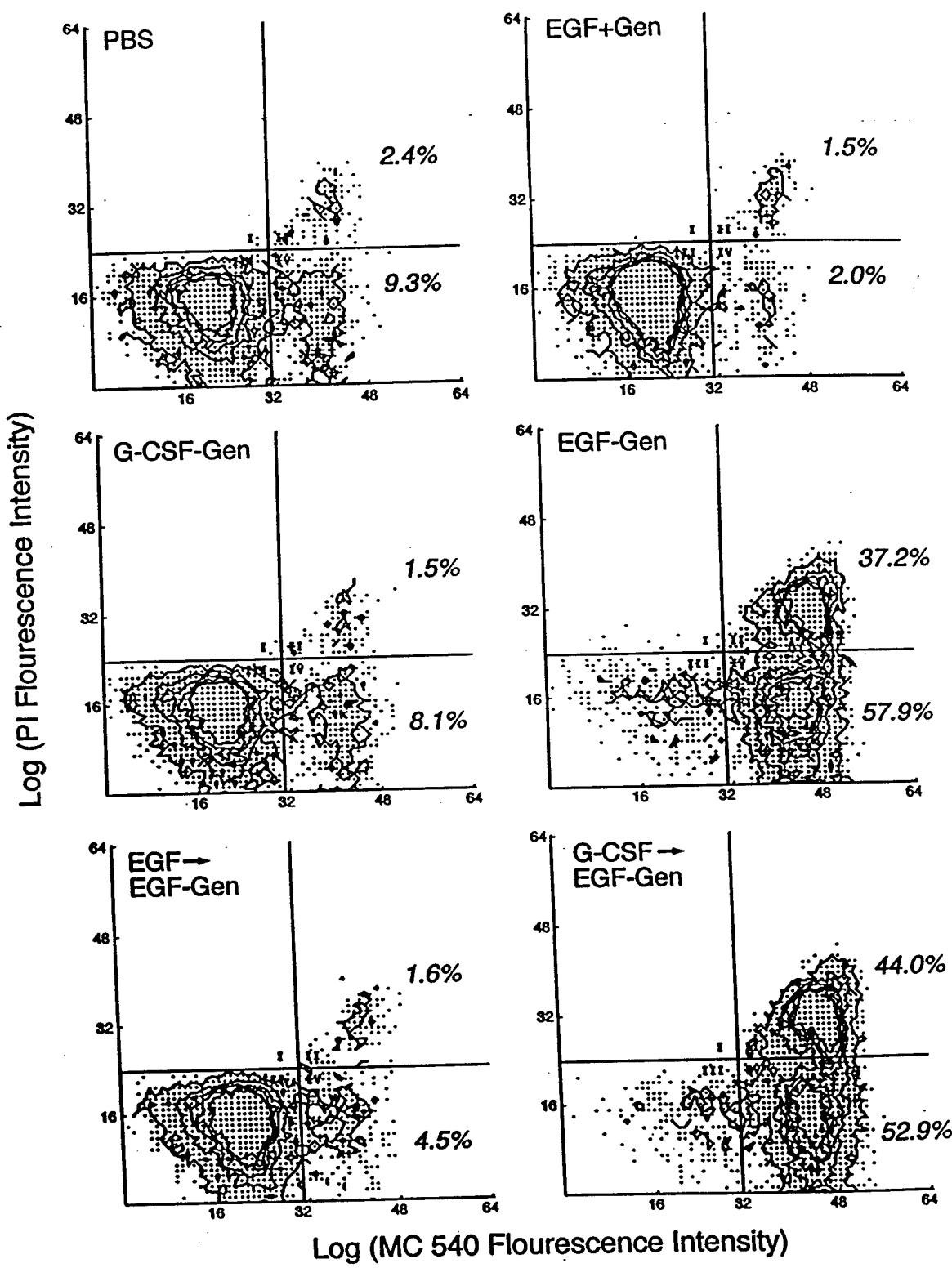


FIGURE 4

## **FIGURE 5**

**EGF-Gen Induces Apoptosis in Human Breast Cancer Cells.** FACS correlated two-parameter displays of MDA-MB-231 cells stained with MC540 and PI 24 hours after treatment with PBS, 10 µg/ml EGF + 10 µg/ml Gen (37 µM), 1 µg/ml G-CSF-Gen, 1 µg/ml EGF-Gen (0.1 µM), 10 µg/ml EGF + 1 µg/ml EGF-Gen, or 10 µg/ml G-CSF + 1 µg/ml EGF-Gen. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence. (see next page)



**FIGURE 5**

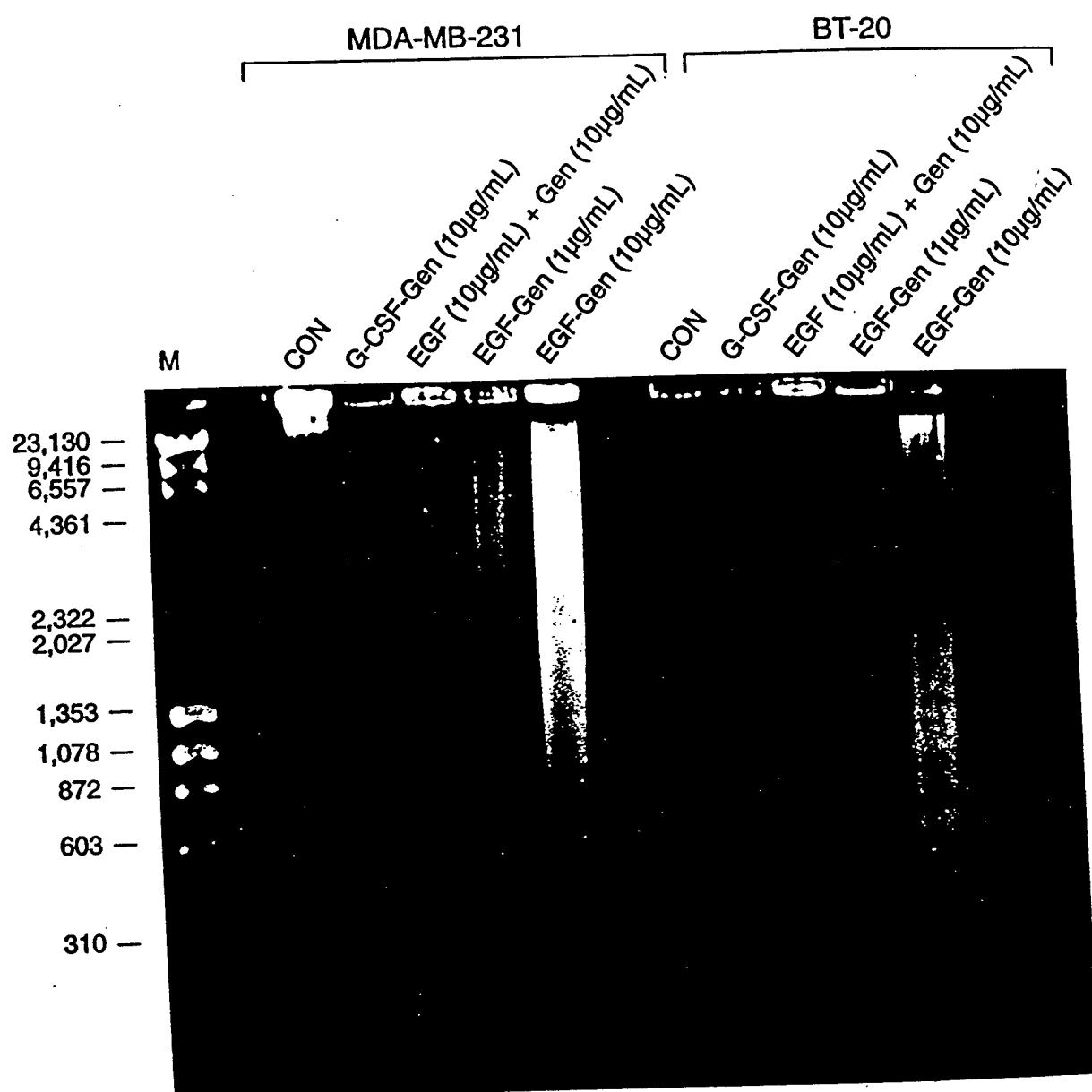
EGF because cells treated with unconjugated Gen plus unconjugated EGF did not show apoptotic DNA fragmentation (Figure 6).

We compared the ability of equimolar concentrations of EGF-Gen and unconjugated Gen to induce apoptosis in dose response studies using the MDA-MB-231 breast cancer cell line. Whereas EGF-Gen caused apoptosis in 98.7 % of treated breast cancer cells at concentrations as low as 0.1  $\mu$ M, Gen was significantly less active and caused apoptosis in only 15.5% of the breast cancer cells even at a 100  $\mu$ M concentration (Figure 7).

We next tested the anti-cancer activity of EGF-Genistein against MDA-B-231 and BT-20 breast cancer cell lines using *in vitro* clonogenic assays. The EGF-R negative leukemia cell line NALM-6 was used as a negative control and the EGF-R positive prostate cancer cell line PC-3 was used as a positive control. As shown in Table 2, 24 hour treatment with 10  $\mu$ g/mL EGF-Gen killed >99% of clonogenic MDA-MB-231 and BT-20 cells as well as >99% of PC-3 cells, under conditions which did not affect the clonogenic growth of EGF-R negative NALM-6 leukemia cells. The lack of toxicity to NALM-6 cells was not caused by a cellular resistance to Gen, because B43-Gen, an anti-CD19 immunoconjugate (18), killed >99% of NALM-6 cells. Unlike EGF-Gen, neither EGF (10  $\mu$ g/mL, unmodified or Sulfo-SANPAH-modified) nor Gen (10  $\mu$ g/mL) were able to inhibit the clonogenic growth of EGF-R positive cancer cell lines. Similarly, G-CSF-Gen (10  $\mu$ g/mL) did not affect the clonogenic growth of these breast and prostate cancer cell lines (Table 2). To more accurately compare the cytotoxic activities of EGF-Gen and unconjugated Gen, we performed detailed dose response studies using *in vitro* clonogenic assays. As shown in Figure 8, EGF-Gen inhibited in each of 3 independent experiments the clonogenic growth of MDA-MB-231 as well as BT-20 cells at nanomolar concentrations with mean IC<sub>50</sub> values of 30±3 nM (Range: 21 - 42 nM) and 30±10 nM (Range: 17-64 nM), respectively (~196 ng/ml), whereas unconjugated Gen elicited substantially less inhibitory activity with >1,000 fold higher mean IC<sub>50</sub> values (120 ± 18  $\mu$ M [Range: 99-154  $\mu$ M] for MDA-MB-231 cells (~32  $\mu$ g/ml) and 112 ± 17  $\mu$ M [Range: 80-139  $\mu$ M] for BT-20 cells (~30  $\mu$ g/ml). The P-values for the Student's t-test comparisons of the IC<sub>50</sub> values for EGF-Gen vs Gen were <0.001 for both cell lines. The IC<sub>50</sub> values derived from the composite

## **FIGURE. 6**

**Internucleosomal DNA Fragmentation in EGF-Gen-Treated Breast Cancer Cells.** Cells were harvested 24 hours after treatment with PBS (CON), EGF-Gen, G-CSF-Gen, or unconjugated EGF + unconjugated Gen, and DNA was prepared for analysis of fragmentation. DNA was then separated by electrophoresis through a 1% agarose gel, and the DNA bands were visualized by UV light after staining with ethidium bromide. Lane M, molecular size markers in base pairs. (see next page)

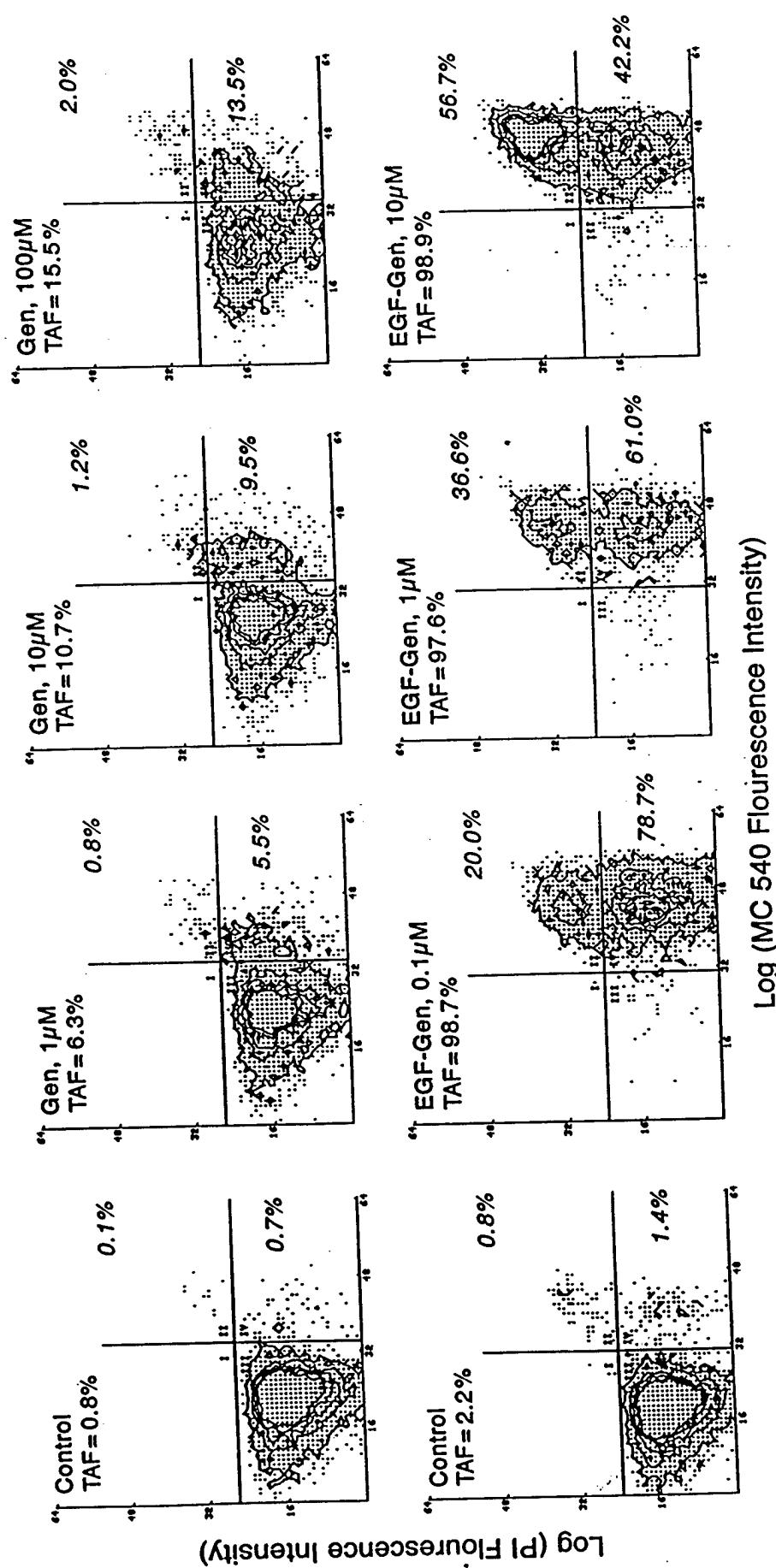


## FIGURE 6

## **FIGURE 7**

**EGF-Gen Induces Apoptosis in Human Breast Cancer Cells.** FACS correlated two-parameter displays of MDA-MB-231 cells stained with MC540 and PI 24 hours after treatment with PBS, EGF-Gen (0.1  $\mu$ M, 1.0  $\mu$ M, 10  $\mu$ M), or unconjugated Gen (1.0  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M). The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence. For each treatment, the total apoptotic fraction (TAF) (= % MC540 single fluorescent + % MC540/PI double fluorescent) is also provided. (see next page)

FIGURE 7



**Table 2. Cytotoxic Activity of EGF-Gen Against Clonogenic Breast Cancer Cells**

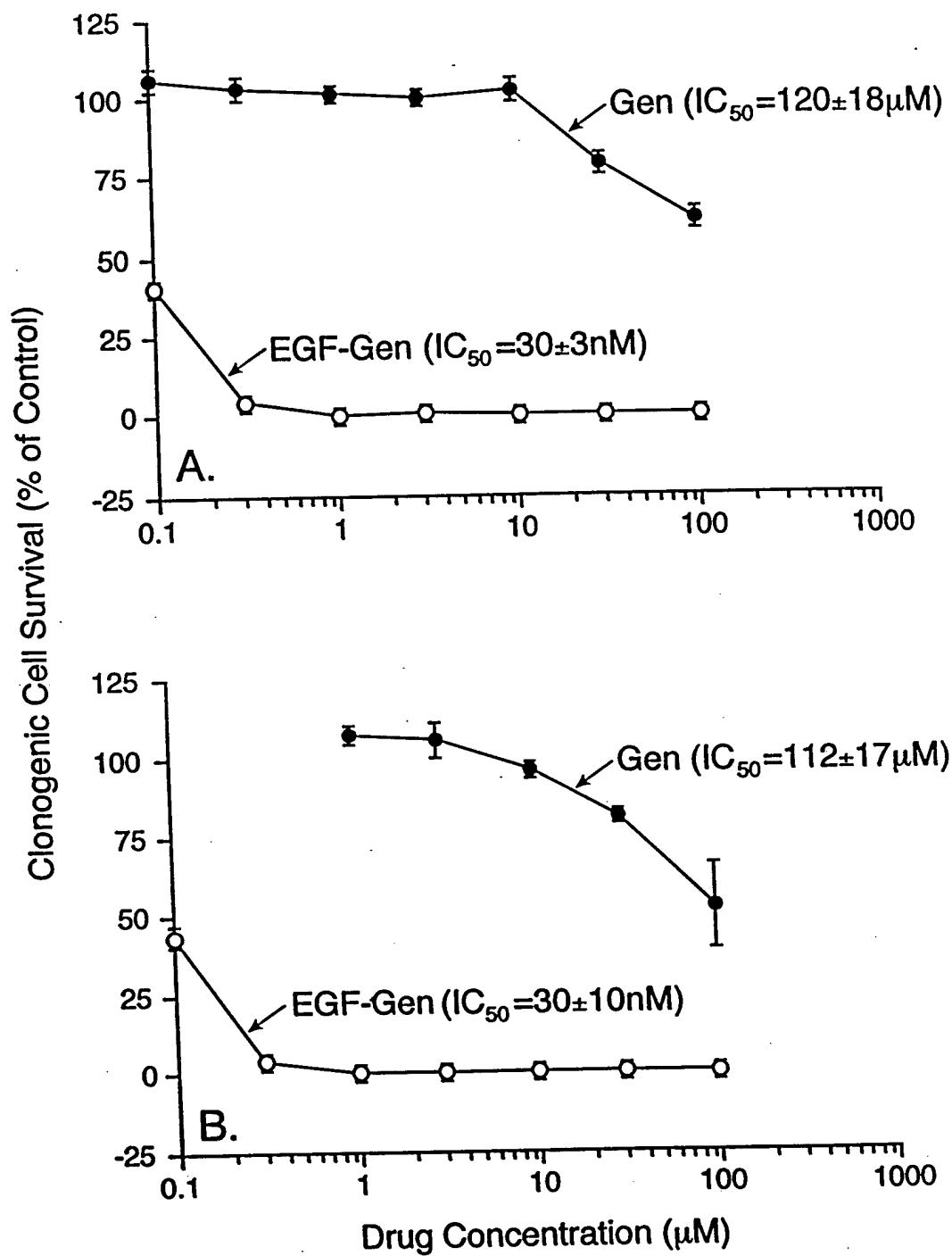
Cell Line	Treatment	Mean No. Colonies Per 1 x 10 <sup>6</sup> Cells	Percent Inhibition of Clonogenic Cells
MDA-MB-231 <i>Breast Cancer</i>	PBS	394 (368,420)	-
	EGF, 10 µg/mL	524 (512,536)	0.0
	Gen, 10 µg/mL	275 (268,282)	30.2
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.4
	GCSF-Gen, 10 µg/mL	395 (390,400)	0.4
BT-20 <i>Breast Cancer</i>	PBS	155 (143,167)	-
	EGF, 10 µg/mL	161 (152,170)	0.0
	Gen, 10 µg/mL	117 (113,121)	24.5
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.4
	GCSF-Gen, 10 µg/mL	154 (150,158)	0.6
PC-3 <i>Prostate Cancer</i>	PBS	298 (287,309)	-
	EGF, 10 µg/mL	355 (307,403)	0.0
	Gen, 10 µg/mL	256 (253,259)	14.1
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.7
	GCSF-Gen, 10 µg/mL	309 (298,320)	0.0
NALM-6 <i>Pre-B ALL</i> EGFR <sub>c</sub> (-)	PBS	214 (209,219)	-
	Gen, 10 µg/mL	175 (162,188)	18.2
	EGF-Gen, 10 µg/mL	210 (187,233)	0.0
	B43-Gen, 10 µg/mL	0 (0,0)	>99.5

Cancer cells were treated with the indicated agents for 24 hours at 37°C, washed twice, and then plated in duplicate Petri dishes at 10<sup>5</sup> cells/mL. The clonogenic medium was alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Colonies were enumerated on day 7 using an inverted phase microscope of high optical resolution.

## **FIGURE 8**

**EGF-Gen is more cytotoxic against clonogenic MDA-MB-231 and BT-20 human breast cancer cells than unconjugated Gen.** MDA-MB-231 (shown in [A]) and BT-20 (shown in [B]) cells were treated with 0.1, 0.3, 1.0, 3.0, 10, 30, or 100  $\mu$ M EGF-Gen or equimolar concentrations of unconjugated Gen for 24 hours. Subsequently, cells were assayed for clonogenic growth in vitro, as described in Materials and Methods. . The colony numbers for MDA-MB-231 cells ranged from 385/ $10^5$  cells to 510 colonies/ $10^5$  cells (mean  $\pm$  SE =  $450 \pm 36$  colonies/ $10^5$  cells). The colony numbers for BT-20 cells ranged from 193/ $10^5$  cells to 276 colonies/ $10^5$  cells (mean  $\pm$  SE =  $224 \pm 26$  colonies/ $10^5$  cells). Composite clonogenic cell survival curves were generated using the dose response data from 3 independent experiments, each performed in duplicate. Each data point on the composite survival curve represents the mean % control clonogenic cell survival at a given drug concentration. The error bars for each data point are the standard error to the mean. The individual IC<sub>50</sub> values for EGF-Gen from the 3 experiments ranged from 21nM to 42nM for MDA-MB-231 cells (mean  $\pm$  SE =  $30 \pm 3$  nM), and 17nM to 64 nM for BT-20 cells (mean  $\pm$  SE =  $30 \pm 10$  nM). The corresponding IC<sub>50</sub> values for Gen ranged from 99  $\mu$ M to 154

$\mu\text{M}$  for MDA-MB-231 cells (mean  $\pm$  SE =  $120 \pm 18 \mu\text{M}$ ), and from  $80 \mu\text{M}$  to  $139 \mu\text{M}$  for BT-20 cells (mean  $\pm$  SE =  $112 \pm 17 \mu\text{M}$ ). The EGF-Gen IC<sub>50</sub> values derived from the composite MDA-MB-31 and BT-20 clonogenic cell survival curves were 39 nM and 20 nM, respectively. By comparison, the IC<sub>50</sub> values of the composite clonogenic survival curves for unconjugated Gen were 160  $\mu\text{M}$  and 147, respectively. (see next page)



**FIGURE 8**

MDA-MB-31 clonogenic cell survival curves were 39 nM for EGF-Gen and 160  $\mu$ M for unconjugated Gen. The IC<sub>50</sub> values derived from the composite BT-20 clonogenic cell survival curves were 20 nM for EGF-Gen and 147  $\mu$ M for unconjugated Gen (Figure 8).

### C. SUMMARY

The receptor (R) for epidermal growth factor (EGF) is expressed at high levels on human breast cancer cells and associates with ErbB2, ErbB3, and Src protooncogene family protein tyrosine kinases (PTK) to form membrane-associated PTK complexes with pivotal signaling functions. Recombinant human EGF was conjugated to the soybean-derived PTK inhibitor genistein (Gen) to construct an EGF-R-directed cytotoxic agent with PTK inhibitory activity. The EGF-Gen conjugate was capable of binding to and entering EGF-R-positive MDA-MB-231 and BT-20 breast cancer cells (but not EGF-R-negative NALM-6 or HL-60 leukemia cells) via its EGF moiety and it effectively competed with unconjugated EGF for target EGF-R molecules in ligand binding assays. EGF-Gen inhibited the EGF-R tyrosine kinase in breast cancer cells at nanomolar concentrations with an IC<sub>50</sub> value of 2.9 nM, whereas the IC<sub>50</sub> value for unconjugated Gen was >100  $\mu$ M. Notably, EGF-Gen triggered a rapid apoptotic cell death in MDA-MB-231 as well as BT-20 breast cancer cells at nanomolar concentrations. The EGF-Gen-induced apoptosis was EGF receptor-specific because cells treated with the control granulocyte-colony stimulating factor (G-CSF)-Gen conjugate did not become apoptotic. Apoptosis was dependent both on the PTK inhibitory function of Gen and the targeting function of EGF because cells treated with unconjugated Gen plus unconjugated EGF did not undergo apoptosis. The IC<sub>50</sub> values of EGF-Gen versus unconjugated Gen against MDA-MB-231 and BT-20 cells in clonogenic assays were  $30 \pm 3$  nM versus  $120 \pm 18$   $\mu$ M ( $P < 0.001$ ) and  $30 \pm 10$  nM versus  $112 \pm 17$   $\mu$ M ( $P < 0.001$ ), respectively. Thus, the EGF-Gen conjugate is a >1,000-fold more potent inhibitor of EGF-R tyrosine kinase activity in intact breast cancer cells than unconjugated Gen and a >1,000-fold more potent cytotoxic agent against EGF-R<sup>+</sup> human breast cancer cells than unconjugated Gen. Taken together, these results indicate that the EGF-R-associated PTK complexes have vital anti-apoptotic functions in human breast cancer cells and may therefore be used as therapeutic targets.

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## **II.2. IN VIVO STUDIES**

### **A. EXPERIMENTAL METHODS**

**Preparation of the EGF-Genistein Conjugate.** EGF-Gen was produced by conjugating recombinant human EGF to genistein (Gen) according to a recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL), as previously described in detail (18). The chemical composition and *in vitro* biologic activity of EGF-Gen were previously reported (18).

**Cross-reactivity of Human EGF and Anti-Human EGF-R Antibodies with Mouse EGF-R.** Livers and thymus of BALB/c mice were frozen in liquid nitrogen and 5 µm thick tissue sections were prepared using a cryostat. The sections were fixed in 2% paraformaldehyde (pH 7.4) and processed for standard indirect immunofluorescence using a monoclonal antibody directed to the extracellular domain of the human EGF-R (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) as the primary antibody and a goat anti-mouse IgG conjugated to FITC (Amersham Corp., Arlington Heights, IL) as the secondary antibody. In parallel, sections were also stained by direct immunofluorescence staining techniques with FITC-conjugated EGF (Molecular Probes, Inc., Eugene, OR) in Hank's Balanced Salt buffer containing BSA, 0.1% sodium azide, and 20 mM HEPES (pH 7.0) according to the manufacturer's recommendations. Coverslips were mounted with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA) to stain the nuclei.

**Mouse Toxicity Studies.** The toxicity profile of EGF-Gen in BALB/c mice was examined, as previously reported for other biotherapeutic agents (19, 21). All BALB/c mice used in the toxicity studies were obtained from the specific pathogen free (SPF) breeding facilities of the National Institutes of Health (NIH; Bethesda, MD) at 6 - 8 weeks of age. The mice were housed in an American Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved specific pathogen-free mouse facility. All husbandry and experimental contact made with the mice maintained SPF conditions.

The mice were kept in Micro-Isolator cages (Lab Products, Inc., Maywood, NY) containing autoclaved food, water and bedding. Female BALB/c mice were used and monitored daily for lethargy, cleanliness and morbidity. At the time of death, necropsies were performed and the toxic effects of immunoconjugate administration were assessed. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 6 micron tissue sections were prepared and stained with Hematoxylin and Eosin (H&E).

In single dose toxicity studies, female BALB/c mice were administered an i.p. bolus injection of EGF-Gen in 0.2 ml PBS, or 0.2 ml PBS alone (control mice). In cumulative toxicity studies, mice received a total of 2800 µg (=140 mg/kg) EGF-Gen i.p. over 28 consecutive days. No sedation or anesthesia was used throughout the treatment period. Mice were monitored daily for mortality for determination of the day 30 LD<sub>50</sub> values. Mice surviving until the end of the 30 days monitoring were sacrificed and the tissues were immediately collected from randomly selected mice, and preserved in 10% neutral buffered formalin. Standard tissues collected for histologic evaluation included: bone, bone marrow, brain, cecum, heart, kidney, large intestine, liver, lung, lymph node, ovary, pancreas, skeletal muscle, skin, small intestine, spleen, stomach, thymus, thyroid gland, urinary bladder, and uterus (as available).

**Breast Cancer Cells.** MDA-MB-231 (ATCC HTB-26) is an EGF-R positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51 year old patient. MDA-MB-231 cell line was maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum. For subculturing, medium was removed from the flasks containing a confluent layer of cells, and fresh 0.25% trypsin was added for 1-2 min. Trypsin was removed and cultures incubated for 5-10 min at 37°C until cells detached. Fresh medium was then added, cells aspirated and dispensed into new flasks.

In some experiments, the cytotoxic activity of plasma samples from EGF-Gen treated cynomolgus monkeys was examined using a methylcellulose colony assay system (18, 22). In brief, MDA-MB-231 cells ( $10^7$ /mL in RPMI + 10% FBS) were treated overnight at 37°C with 1: 20 (v/v) PBS-diluted plasma samples from EGF-Gen-treated monkeys. After treatment, cells were washed twice, plated at  $10^6$

cells/mL in RPMI + 10% FBS + 0.9% methylcellulose in Petri dishes, and cultured for 7 days at 37°C in a humidified 5% CO<sub>2</sub> incubator. Subsequently, MDA-MB-231 colonies containing > 20 cells were enumerated using a inverted phase-contrast microscope and the % inhibition of colony formation was calculated using the formula: % Inhibition = 1 - (Mean No. Colonies in Test Culture/Mean No. Colonies in Control Culture)×100. In some experiments, excess unconjugated EGF was added to the plasma samples to block the action of EGF-Gen by competing for the EGF-R on cancer cells. Excess G-CSF was used as a control.

**Maintenance of SCID Mouse Colony.** The SCID mice were housed in an AAALAC-approved specific pathogen-free facility. Animal housing was located in a secure indoor facility with controlled temperature, humidity, and noise levels. The SCID mice were housed in microisolater cages which were autoclaved with rodent chow. Water was also autoclaved and supplemented with trimethoprim/sulfomethoxazol 3 days/week.

**SCID Mouse Xenograft Model of Human Breast Cancer.** The left hind legs of the CB. 17 SCID mice were inoculated s.c. with 1×10<sup>6</sup> MDA-MB-231 breast cancer cells in 0.2 mL PBS. SCID mice inoculated with human breast cancer cells were treated with EGF-Gen (0.2 µg/dose = 10 µg/kg/dose or 2.0 µg/dose = 100 µg/kg/dose in 0.2 ml PBS) with daily i.p doses for 10 treatment days starting the day after inoculation of cancer cells. Daily treatments with PBS, 10 µg (=500 µg/kg) G-CSF-Gen, 10 µg Gen (=500 µg/kg) combined with 10 µg (500 µg/kg) EGF or 10 µg (500 µg/kg) Gen alone were used as controls. 50 µg (2.5 mg/kg) Adriamycin (Ben Venue Laboratories, Inc., Bedford, OH 44146) or 9.3 µg (= 465 µg/kg) methotrexate (Lederle Parenterals, Inc., Carolina, Puerto Rico 00630) were given as single dose i.p. bolus injections on the day after inoculation of cancer cells. 1 mg (50 mg/kg) cyclophosphamide (Bristol-Myers Squibb Co., Princeton, New Jersey 08543) was injected i.p. on two consecutive days starting the day after inoculation of cancer cells. Mice were monitored daily for health status and tumor growth, and were sacrificed if they became moribund, developed tumors which impeded their ability to attain food or water, or at the end of the 7-month observation period. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded

in paraffin by routine methods. Glass slides with affixed 6 micron tissue sections were prepared and stained with hematoxylin/eosin. Primary endpoints of interest were tumor growth and tumor -free survival outcome. Estimation of life table outcome and comparisons of outcome between groups were done, as previously reported (19-21). The efficacy of EGF-Gen against established tumors was examined by treating SCID mice with subcutaneous MDA-MB-231 xenografts of 0.5 cm or 1.0 diameter with 100 µg/kg/day EGF-Gen i.p on 10 consecutive days and determining the tumor diameter daily for 20 days from the start of therapy. Control mice were treated with 0.2 ml PBS for 10 consecutive days.

**Cynomolgus Monkey Experiments.** Female cynomolgus monkeys were obtained from BioMedical Resources Foundation of Houston, TX. The monkeys were housed in the AAALAC-accredited primate facility. The monkeys were singly housed in stainless steel cages and provided with toys and treats for enrichment. Prior to entering the study, the monkeys were housed in a quarantine-room in the same facility for 6 weeks. During this time, they were TB-tested three times, serologically screened for Herpes virus simiae, and screened for enteric bacterial, protozoal, and helminth pathogens. In pharmacodynamic studies, monkeys were fasted overnight prior to anesthesia and treatment. After induction of anesthesia (Ketamine hydrochloride 10-15 mg/kg), a catheter was placed percutaneously either into the right or left cephalic vein using a sterile disposable kit. This catheter was taped in place for administration of EGF-Gen or maintenance fluids (Normal saline at 4 ml/kg/hr via an infusion pump) and for drawing of blood samples. A Harvard infusion pump was used to administer EGF-Gen as a constant intravenous infusion over a 1 hour period.

**Pharmacokinetic Studies.** Tissue distribution studies in SCID mice were performed using EGF-<sup>125</sup>I-Gen and <sup>125</sup>I-Gen, as described in detail in previous publications from our laboratory (19). A flow-limited physiological pharmacokinetic model was used to characterize the tissue disposition of EGF-Gen in non-tumor bearing as well as tumor bearing SCID mice (19, 23). Tissue volumes and plasma flow rates were those previously described for mice (24). A set of linear differential equations describing the mass balances of each model compartment was used to estimate tissue partition coefficients (i.e., the ratio of the drug concentration in the tissue of interest to the drug concentration in the plasma at equilibrium)

for each organ. These differential equations were simultaneously solved using the ADAPT II software (25). Biliary excretion and gut reabsorption were incorporated into the physiological model in the form of saturable processes, based on previous studies establishing saturable biliary excretion of unconjugated recombinant human EGF (26). In plasma half-life studies in SCID mice and cynomolgus monkeys, the EGF-Gen conjugate concentrations were measured in the plasma samples using the EGF Quantikine ELISA kit from R&D Systems, a quantitative sandwich enzyme immunoassay, which allows the detection of the EGF-Gen conjugate via its EGF moiety. In these studies, EGF-Gen was administered to SCID mice by intraperitoneal injection at doses of 100 µg/kg and 1 mg/kg. Four mice were used at each dose level, and blood samples were obtained at six non-overlapping time points from each pair of mice. Mice were serially bled by retroorbital puncture at 0 min, 10 min, 30 min, 1 hour, 2 hours, 4 hours, and 12 hours following the administration of the first bolus dose of EGF-Gen. In cynomolgus monkeys, EGF-Gen was administered on each of the 10 treatment days intravenously over 1 hour and plasma samples were obtained at 0 min, 30 min, 45 min, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours post-infusion time points after the first dose of EGF-Gen. A two compartment first-order pharmacokinetic model was fit to the plasma concentration-versus-time data for EGF-Gen. Maximum likelihood estimation as implemented in ADAPT II software, was used to estimate the central compartment volume of distribution, elimination rate constant, and distribution rate constants for EGF-Gen, as previously described (19-21, 25).

## B. RESULTS

**Biodistribution and Toxicity of EGF-Gen in Mice.** Human EGF binds to murine EGF-R in mouse tissues, as determined by immunocytochemistry (Figure 9). Because of the crossreactivity of human EGF and EGF-Gen with murine EGF-R, we decided to use mice in the initial evaluation of the biodistribution and toxicity of EGF-Gen. Tissue distribution studies were performed using EGF-<sup>125</sup>I-Gen and a flow-limited physiological pharmacokinetic model was used to characterize the *in vivo* tissue disposition of EGF-Gen in non-tumor bearing as well as tumor bearing SCID mice. For each organ, the partition coefficients (i.e., R values = the tissue-to-plasma equilibrium distribution ratios for linear

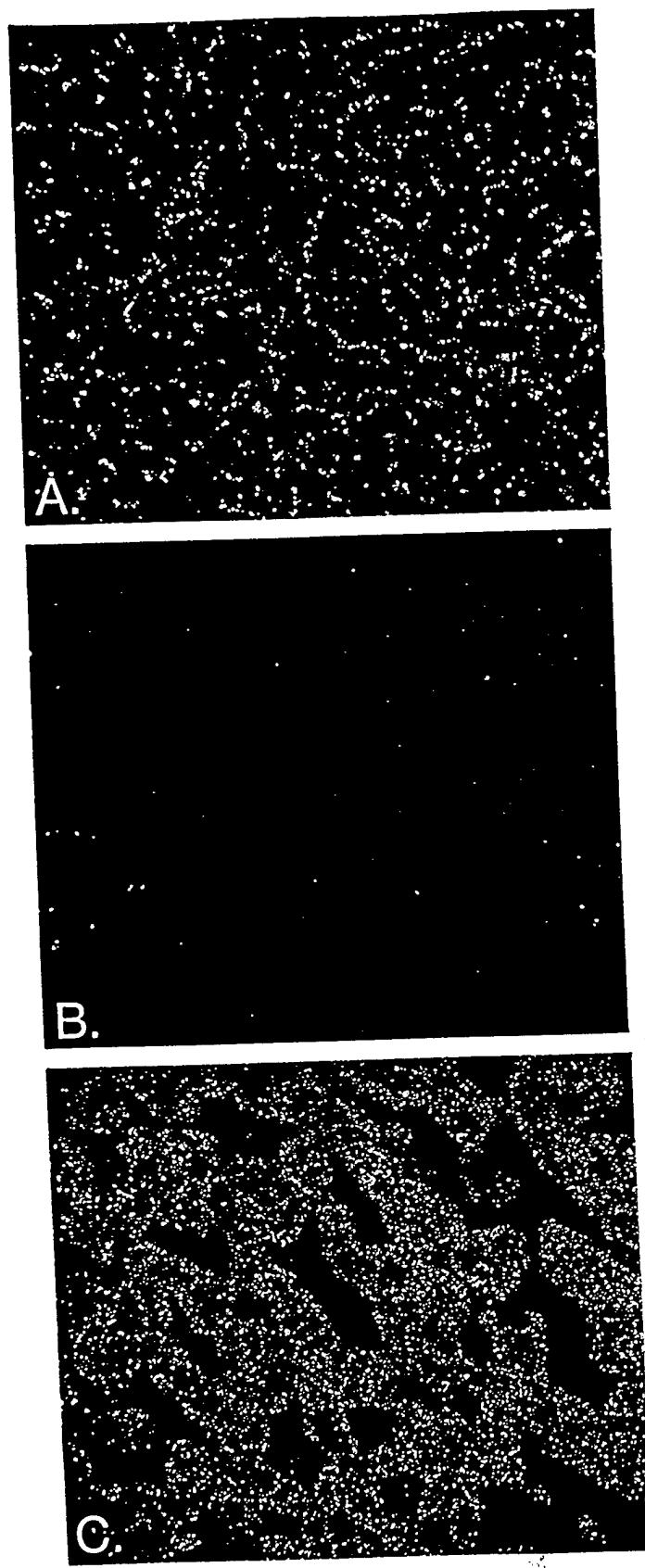
binding) were determined, as previously described (19). When compared to unconjugated Gen, a much greater amount of EGF-Gen partitioned to bone marrow (R bone marrow: 0.00008 ml/g for Gen vs 0.53 ml/g for EGF-Gen), spleen (R spleen: 0.04 ml/g for Gen vs 5.28 ml/g for EGF-Gen), liver (R liver: 0.50 ml/g for Gen vs 7.40 ml/g for EGF-Gen), kidney (R kidney: 0.25 ml/g for Gen vs 0.93 ml/g for EGF-ml/g for Gen vs 7.40 ml/g for EGF-Gen), and lungs (R lungs: 0.53 ml/g for Gen vs 1.44 ml/g for EGF-Gen) (Table 3). In both tumor bearing and non-tumor bearing mice, EGF-Gen most extensively partitioned to the liver with tissue drug concentrations exceeding plasma concentrations more than seven times ( $P<0.05$ ) (Table 3). By contrast, very little EGF-Gen partitioned to the subcutaneous xenografts in tumor bearing SCID mice. The partition coefficients for the liver and tumor were 8.2 and 0.2, respectively (Table 3).

In toxicity studies, 28 female BALB/c mice were injected intraperitoneally with a single bolus dose of 2  $\mu\text{g}$  ( $=100 \mu\text{g}/\text{kg}$ ) - 800  $\mu\text{g}$  ( $=40 \text{ mg}/\text{kg}$ ) EGF-Gen in 0.2 ml PBS. EGF-Gen was not toxic to mice at any of these dose levels; none of the mice experienced any side effects or died of toxicity during the 30 day observation period. Even at the highest doses of 400  $\mu\text{g}$  or 800  $\mu\text{g}$  ( $=40 \text{ mg}/\text{kg}$ ) EGF-Gen, mice did not become weak or lethargic, lose weight, or develop diarrhea or a scruffy skin. When mice were treated with multiple doses of EGF-Gen at a total dose level of 2.8 mg ( $=140 \text{ mg}/\text{kg}$ ) according to a 100  $\mu\text{g}/\text{mouse/day}$  ( $=5 \text{ mg}/\text{kg/day}$ )  $\times$  28 days schedule, no significant toxicity was observed and none of the 10 mice died. No histopathologic lesions were found in any of the organs of EGF-Gen treated mice receiving a single dose or multiple doses of EGF-Gen, including the liver which had the highest partition coefficient in tissue distribution studies. Thus, the maximum tolerated dose (MTD ~ LD10) of EGF-Gen was not reached at the 40  $\text{mg}/\text{kg}$  single dose level or the 140  $\text{mg}/\text{kg}$  cumulative dose level.

***In Vivo* Anti-tumor Activity of EGF-Gen in a SCID Mouse Xenograft Model of Human Breast Cancer.** CB.17 SCID mice developed rapidly growing tumors after subcutaneous inoculation of  $1 \times 10^6$  MDA-MB-231 cells. We examined the *in vivo* anti-tumor activity of EGF-Gen in this SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a dose-dependent fashion, when it was administered 24 hours after inoculation of tumor cells. At a dose level of 100  $\mu\text{g}/\text{kg}/\text{d} \times 10$  days (1  $\text{mg}/\text{kg}$  total dose), which is  $>100$ -fold less than the highest tested and

## **FIGURE 9**

**Binding of Anti-Human EGF-R Antibody and Human EGF to EGF-R on Murine Hepatocytes.** [A] Expression of EGF-R (green fluorescence) on the hepatocytes as shown by crossreactivity of anti-human EGF-R antibody. [B] Lack of binding of FITC-conjugated human EGF (green fluorescence) to murine thymocytes. [C] Binding of FITC-conjugated human EGF (green fluorescence) to murine hepatocytes. Red fluorescence represents the propidium iodide staining of the nuclei. (see next page)



**FIGURE 9**

**Table 3. Tissue Distribution Parameters for EGF-Genistein and Genistein in SCID Mice.**

Tissue	EGF-Gen		Gen
	No Tumor	Tumor	No Tumor
<i>Linear binding constant (R)</i>			
Brain	0.05	0.05	0.03
Heart	0.38	0.35	0.34
Skin	0.20	0.15	0.21
Muscle	0.11	0.09	0.09
Bone marrow	0.53	0.42	0.00008
Stomach	1.00	1.13	N.D.
Spleen	5.28	3.87	0.04
Lungs	1.44	0.84	0.53
Kidney	0.93	0.86	0.25
Liver	7.40	8.15	0.50
Intestine	0.86	1.08	N.D.
Tumor	N.A.	0.16	N.A.

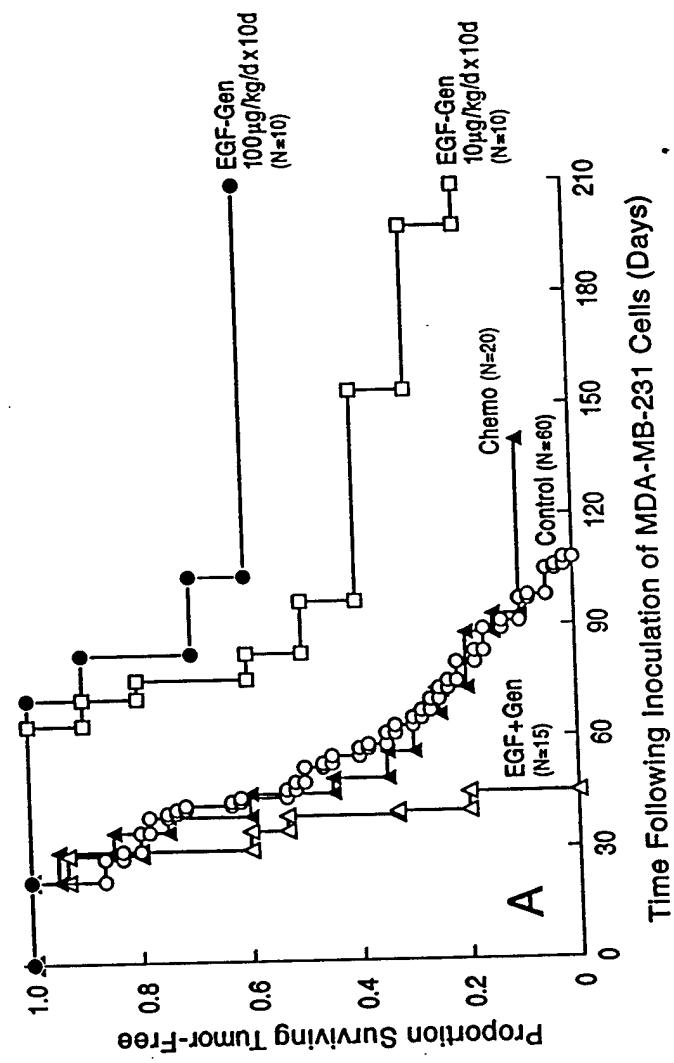
A flow-limited physiological pharmacokinetic model was used to characterize the tissue disposition of both drugs in mice. Volume terms and flow rates were those previously described for mice (23). A set of differential equations describing the mass balances of each model compartment was used to estimate linear binding constants for each organ. These differential equations were simultaneously solved with the use of ADAPT II software (25). R, tissue-to-plasma equilibrium distribution ratio for linear binding in ml/g. N.A., not applicable.

nontoxic cumulative dose (i.e., 140 mg/kg) in mice, EGF-Gen was more effective than cyclophosphamide (50 mg/kg/d x 2 days), adriamycin (2.5 mg/kg x 1) or methotrexate (0.5 mg/kg x 1), the most widely used standard chemotherapeutic drugs for breast cancer. Figure 10 shows the tumor-free survival outcome of SCID mice treated with EGF-Gen, chemotherapeutic drugs (CHEMO = cyclophosphamide, adriamycin, or methotrexate), or control agents after inoculation with MDA-MB-231 breast cancer cells. None of the 60 control mice (CON) treated with PBS (N=40), G-CSF-Gen (100 µg/kg/day ) (N=10), or unconjugated Gen (100 µg/kg/day) (N=10) remained alive tumor-free beyond 108 days (median tumor-free survival = 52 days) (Figure 10A). All of the 10 mice treated with EGF plus Gen developed tumors within 45 days with a median tumor-free survival of only 39 days. Tumors reached a size of 0.5 cm<sup>3</sup> by 84±3 days in PBS, G-CSF-Gen, or Gen treated CON mice, 62± 1 days in EGF plus Gen treated mice, and 77 ± 16 days in the CHEMO group (Figure 10B). Within the CHEMO group, all of the adriamycin-treated mice developed rapidly growing tumors with a median tumor-free survival time of only 42 days. Tumors reached a size of 0.5 cm<sup>3</sup> by 92 ± 6 days in cyclophosphamide-treated mice, 72 ± 5 days in adriamycin treated mice, and 71 ± 5 days in methotrexate-treated mice. By comparison, 40 ± 16% of mice treated for 10 consecutive days with 10 µg/kg/day EGF-Gen survived tumor-free beyond 3 months and 20 ± 13% were still alive tumor-free at 7 months (median tumor-free survival time = 97 days; CON vs 10 µg/kg/day EGF-Gen, P<0.0001 by log-rank test). Remarkably, 60 ± 16% of mice treated for 10 consecutive days with 100 µg/kg/day EGF-Gen remained alive free of detectable tumors for more than 7 months (CON vs 100 µg/kg/day EGF-Gen, P<0.00001 by log-rank test) (Figure 10). Tumors developing in EGF-Gen-treated mice reached the 0.5 cm<sup>3</sup> tumor size much later than control mice (114 ± 8 days [10 µg/kg/day dose level] and 129 ± 14 days [100 µg/kg/day dose level] vs 84± 3 days, P=0.007 (10 µg/kg/day dose level) and P<0.001 (100 µg/kg/day dose level)). The average size (mean ± SE) of tumors at 90 days and 120 days were 0.257±0.059 cm<sup>3</sup> and 0.822±0.146 cm<sup>3</sup>, respectively for mice in the CON group. By comparison, the average size (mean ± SE) of tumors at 90 days and 120 days were significantly smaller at 0.013± 0.007 cm<sup>3</sup> (P=0.009) and 0.166 ± 0.083 cm<sup>3</sup> (P=0.006) for mice treated with EGF-Gen at 100 µg/kg/day dose level. Thus, EGF-Gen elicited significant *in vivo* anti-tumor activity at non-toxic doses. The inability of 10 µg (=500 µg/kg)/day x 10 days of unconjugated Gen (= 37,000 pmols) in combination with unconjugated EGF to confer tumor-free

## **FIGURE 10**

**Antitumor Activity of EGF-Gen Against Human Breast Cancer in SCID Mice.** Tumor free survival curves (shown in A) and life-table analysis of tumor-free survival outcome (shown in B) of SCID mice challenged with  $1 \times 10^6$  MDA-MB-231 cells. Twenty-four hours after s.c. inoculation of cancer cells, mice received EGF-Gen (10  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days, N=10, or 100  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days, N=10), EGF (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days) + Gen (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days) (N=15), or chemotherapy (N=20) (i.e., cyclophosphamide , 50 mg/kg/day x 2 days; adriamycin, 2.5 mg/kg single bolus dose; or methotrexate, 0.5 mg/kg single bolus dose), as described in Materials and Methods. Controls (N=60) were treated with PBS, G-CSF-Gen (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days), or Gen (500  $\mu\text{g}/\text{kg}/\text{day} \times 10$  days). <sup>1</sup> The P-values for tumor-free survival comparisons were determined using the log-rank test, whereas the P-values for the average time to 0.5 cm<sup>3</sup> tumor size were determined using student t-tests. (see next page)

**FIGURE 10**



### B. Life Table Analysis

Treatment Group	# of Mice	Proportion Surviving Tumor-Free (%)			Median Tumor-Free Survival (days)	P-value (log rank) vs PBS	P-value (log rank) vs EGF-Gen 2 μg/day	Time to 0.5cm <sup>3</sup> tumor size	P-value vs PBS
		30 days	60 days	90 days					
CON	60	83±5	35±6	13±4	0±0	52	—	<0.00001	—
EGF+Gen	15	80±10	0±0	0±0	0±0	39	0.003 <sup>1</sup>	<0.0001	62±1
EGF-Gen, 10 μg/kg/day	10	100±0	100±0	50±16	30±15	97	<0.00001	0.2	114±8
EGF-Gen, 100 μg/kg/day	10	100±0	100±0	70±15	60±16	>210	<0.00001	—	129±14
CHEMO	20	95±5	30±10	15±8	N.D.	48	NS	<0.0001	77±16

survival in this SCID mouse model in contrast to the potency of 2  $\mu$ g (=100  $\mu$ g/kg)/day  $\times$  10 days EGF-Gen containing 309 pmols of Gen demonstrates that (a) the in vivo anti-tumor activity of EGF-Gen cannot be attributed to its EGF moiety alone and (b) conjugation to EGF enhances the anti-tumor activity of Gen against breast cancer cells by >100-fold.

In contrast to EGF-Gen, cyclophosphamide (50 mg/kg/day  $\times$  2 days; N=5), adriamycin (2.5 mg/kg, N=10), or methotrexate (0.5 mg/kg, N=5) did not significantly affect tumor development in this SCID mouse model. Of the 20 mice treated with one of these chemotherapeutic drugs, only 10 $\pm$ 7 % remained tumor-free beyond 3 months, which indicates no improvement over the control group and a worse tumor-free survival outcome compared to the 2.0  $\mu$ g/day EGF-Gen group (median tumor-free survival = 48 days; CON vs CHEMO, P= 0.32; EGF-Gen, 2.0  $\mu$ g/day vs CHEMO, P<0.05) (Figure 10B)

Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in eradication of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days. The day 10 diameters of tumors in the EGF-Gen treated group were 0.1 cm, 0.0 cm, 0.0 cm, 0.2 cm, and 0.1 cm with a mean ( $\pm$  SE) diameter of 0.08  $\pm$  0.04 cm (Figure 11A). There was no significant tumor progression between days 10 and 20 in this group of mice. The day 20 tumor diameters were 0.3 cm, 0.0 cm, 0.0 cm, 0.1 cm, and 0.2 cm with a mean ( $\pm$ SE) diameter of 0.12  $\pm$  0.06 cm. In contrast to the tumors in EGF-Gen-treated mice, all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day  $\times$  10 days ) showed >200% increase in diameter within 10 days: The day 10 tumor diameters ranged from 1.0 to 1.4 cm (mean  $\pm$ SE = 1.24  $\pm$  0.08 cm) in PBS-treated mice and from 1.2 to 1.4 cm (mean  $\pm$ SE= 1.28  $\pm$ 0.04 cm) in Gen-treated mice (P values <0.0001 for EGF-Gen vs PBS as well as EGF-Gen vs Gen). These tumors continued their rapidly progressive growth and the day 20 dimaters ranged from 1.8 to 2.6 cm (mean  $\pm$ SE= 2.20 $\pm$ 0.16 cm) in PBS-treated mice and from 1.9 to 2.4 (mean  $\pm$ SE = 2.12  $\pm$  0.10 cm) in Gen-treated mice (Figure 11A).

EGF-Gen treatment significantly reduced the growth rate of breast cancer xenografts of 1.0 cm diameter during the 20-day observation period but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors (Figure 11B). The day 10 tumor diameters ranged from 2.4 to 3.3 cm (mean  $\pm$  SE =  $2.90 \pm 0.21$  cm) in PBS-treated mice and from 2.3 to 3.5 cm (mean  $\pm$  SE =  $2.90 \pm 0.25$  cm) in Gen-treated mice. By comparison, the tumor diameters in EGF-Gen-treated mice ranged from 1.3 to 2.3 cm (mean  $\pm$  SE =  $1.70 \pm 0.23$  cm) (P values: EGF-Gen vs PBS = 0.008; EGF-Gen vs Gen = 0.009) (Figure 11B).

**In Vivo Pharmacokinetic Features of EGF-Gen in SCID mice.** We sought to determine the therapeutic systemic exposure levels of EGF-Gen by examining its pharmacokinetics when administered at dose levels which were effective in the SCID mouse xenograft model of human breast cancer. Thus, SCID mice were treated with daily intraperitoneal (i.p) bolus injections of 10  $\mu$ g/kg or 100  $\mu$ g/kg EGF-Gen for 10 consecutive days. The differences between various pharmacokinetic parameters of healthy mice versus mice with breast cancer xenografts were calculated based on 95% confidence intervals provided by ADAPT II software. EGF-Gen was cleared rapidly from blood with an elimination half-life of 1.3 ( $\pm 0.2$ )-1.6 ( $\pm 0.4$ ) hours (Table 4, Figure 12A). At the lower dose level, EGF-Gen was cleared more rapidly from blood [13.1 ( $\pm 2.4$ ) vs 6.4 ( $\pm 0.6$ ) ml/hr/g], had a larger central volume of distribution [30.0 ( $\pm 3.6$ ) vs 11.6 ( $\pm 1.3$ ) ml/g], had a lower measured maximum plasma concentration (0.33 vs 0.45 ng/ml), and yielded a lower systemic exposure level (i.e., area under the concentration-time curve) [7.6 ( $\pm 1.4$ ) vs 16.0 ( $\pm 1.5$ )  $\mu$ gxhr/L] in SCID mice with human breast cancer xenografts than in healthy SCID mice that were not inoculated with breast cancer cells ( $P < 0.05$ ) (Table 4, Figure 12A). The differences between these parameters were calculated based on 95% confidence intervals provided by ADAPT II software. These results suggested that EGF-Gen likely binds to EGF-R<sup>+</sup> human breast cancer cells infiltrating SCID mouse tissues, resulting in more rapid removal from plasma in mice with metastatic human breast cancer. The systemic exposure level, as measured by the area under the serum concentration-time curve (AUC), achieved by the therapeutically effective 10  $\mu$ g/kg/day dose level of EGF-Gen was 16.0 ( $\pm 1.5$ )  $\mu$ g\*hr/L in non-tumor bearing healthy SCID mice and 7.6 ( $\pm 1.4$ )  $\mu$ g\*hr/L in SCID mice bearing 1 cm<sup>3</sup> MDA-MB-231 tumors (Table 4). By comparison, treatment with 100

## **FIGURE 11**

***In vivo activity of EGF-Gen against established tumors.*** The efficacy of EGF-Gen against established tumors was examined by treating SCID mice with subcutaneous MDA-MB-231 human breast cancer xenografts of 0.5 cm (shown in [A]) or 1.0 diameter (shown in [B]) with 100 µg/kg/day EGF-Gen i.p for 10 consecutive days and determining the tumor diameter daily for 20 days from the start of therapy. Control mice were treated with 0.2 ml PBS for 10 consecutive days or unconjugated Gen at 500 µg/kg/day x 10 days. P-values were determined using student t-tests. (see next page)

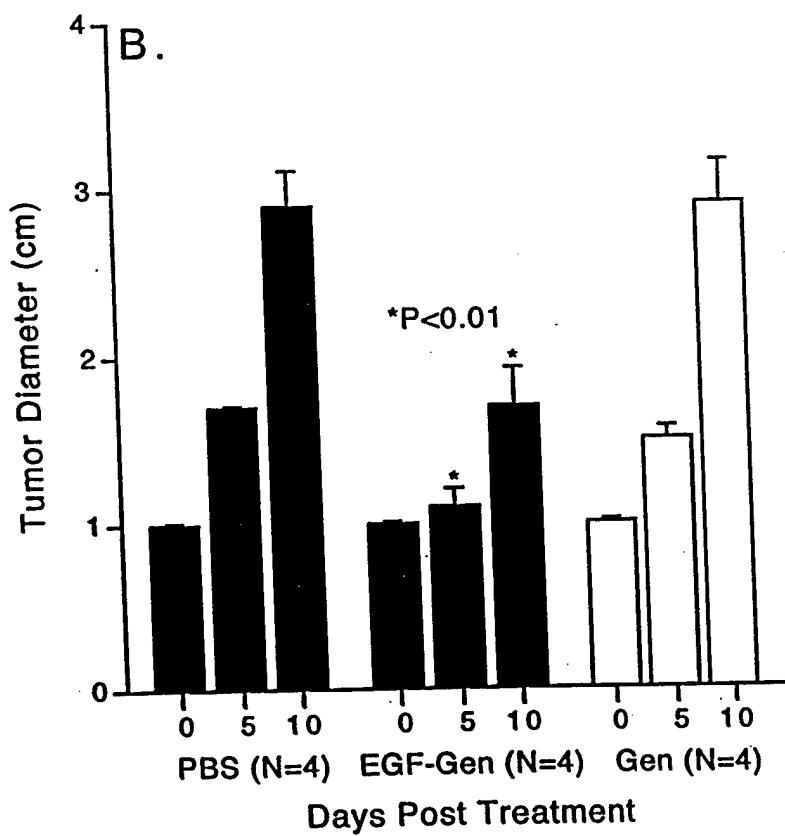
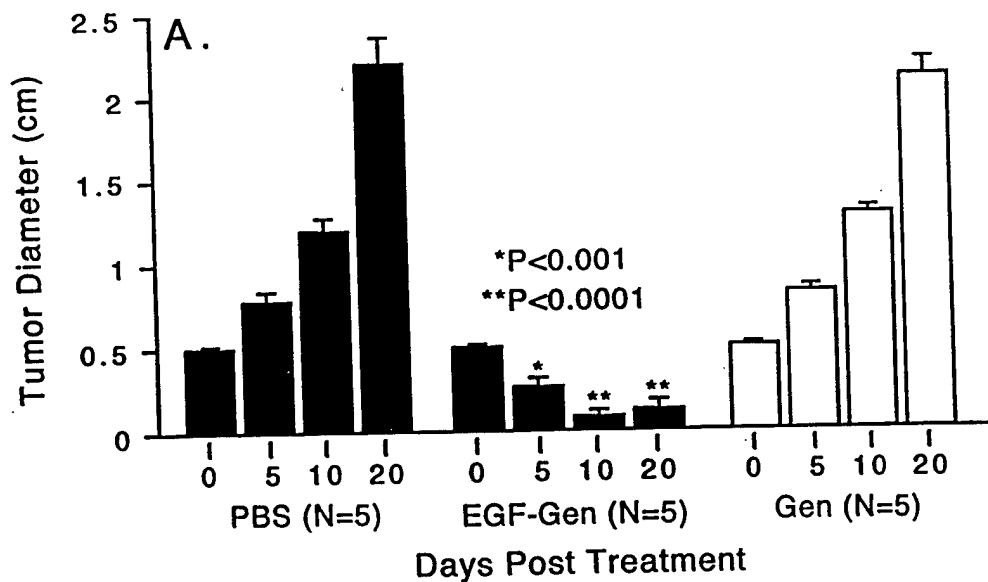


FIGURE 11

## **FIGURE 12**

**Pharmacokinetic Features of EGF-Gen in SCID Mice (Panel A) and Cynomolgus Monkeys (Panel B).** Plasma concentration-time curves of EGF-Gen after intraperitoneal bolus injection into tumor-free SCID mice at doses of 0.1  $\mu\text{g/g}$  (□) and 1.0  $\mu\text{g/g}$  (Δ), and into MDA-MB-231 tumor xenograft-bearing SCID mice at 0.1  $\mu\text{g/g}$  (■) and 1.0  $\mu\text{g/g}$  (▲). Monkeys received a 1-hour intravenous infusion of EGF-Gen at 0.05  $\mu\text{g/g}$  (○) and 0.1  $\mu\text{g/g}$  (□) dose levels. Lines represent pharmacokinetic model simulations for tumor-free (solid line) and tumor-bearing (dashed line) animals; symbols depict measured plasma concentrations of EGF-Gen. (see next page)

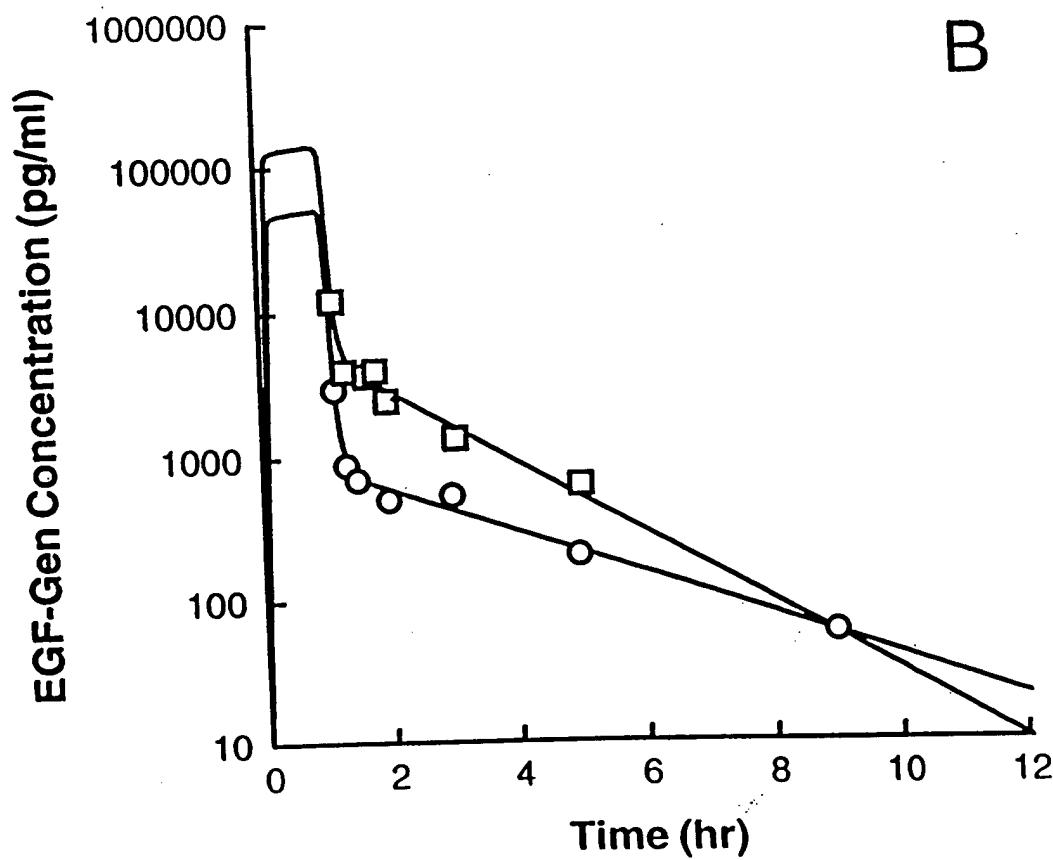
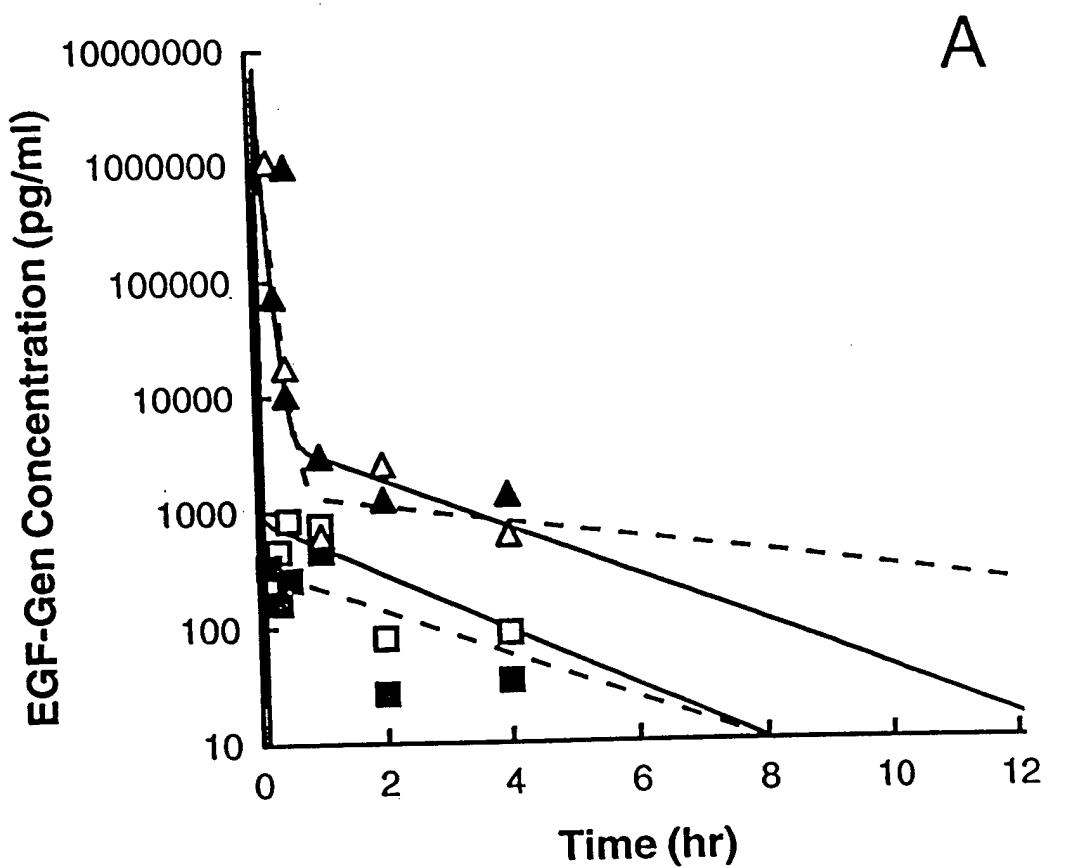


FIGURE 12

**Table 4. Pharmacokinetic Parameters of EGF-Gen in Mice and Monkeys**

Parameter	(units)	Mice without Tumor		Mice with 1 cm <sup>3</sup> Tumor		Monkeys without Tumor	
		100 µg/kg	1,000 µg/kg	100 µg/kg	1,000 µg/kg	500 µg/kg	1,000 µg/kg
Vc	(ml/g)	11.6	0.05	30.0	0.05	0.06	0.04
Ke	(1/hr)	0.6	7.8	0.4	6.5	17.3	16.0
Kcp	(1/hr)	0	0.3	0	0.2	1.0	1.4
Kpc	(1/hr)	0	0.3	0	0.7	0.4	0.6
T <sub>1/2α</sub>	(hr)	0	0.09	0	0.1	0.04	0.04
T <sub>1/2β</sub>	(hr)	1.3	2.1	1.6	1.0	2.1	1.2
CL	(ml/hr/g)	6.4	0.39	13.1	0.32	1.0	0.7
Vdss	(ml/g)	11.6	0.1	30	0.07	0.2	0.7
Cmax	(ng/ml)	0.45	960	0.33	1100	2.9	12.0
AUC	(µg x hr/L)	16	2564	7.6	3125	500	1400

Vc = central volume of distribution; Ke = elimination rate constant; Kcp, Kpc = distribution rate constants, T<sub>1/2α</sub> = distribution half-life, T<sub>1/2β</sub> = elimination half-life; CL = systemic clearance from plasma, Vdss = volume of distribution at steady state, Cmax = measured maximum plasma concentration, AUC = area under the concentration-time curve. Dose 1 µg/g was used for efficacy study.

$\mu\text{g}/\text{kg}/\text{day} \times 10$  days of EGF-Gen yielded an AUC of  $2564 (\pm 231) \mu\text{gxhr/L}$  in non-tumor bearing mice and an AUC of  $3125 (\pm 281) \mu\text{gxhr/L}$  in tumor-bearing mice. Thus, the AUC showed a dramatic 160-fold ( $2564 \mu\text{gxhr/L}$  vs  $16 \mu\text{gxhr/L}$ ,  $P < 0.001$ ) to 411-fold ( $3125 \mu\text{gxhr/L}$  vs  $7.6 \mu\text{gxhr/L}$ ,  $P < 0.001$ ) increase as the dose of EGF-Gen was increased 10-fold. This dramatic increase in AUC which was accompanied by a dramatic 232-fold [ $11.6 (\pm 1.3) \text{ ml/g}$  vs  $0.05 (\pm 0.01) \text{ ml/g}$ ,  $P < 0.001$ ] to 600-fold [ $30.0 (\pm 3.6) \text{ ml/g}$  vs  $0.05 (\pm 0.01) \text{ ml/g}$ ,  $P < 0.001$ ] decrease of the volume of distribution is most likely due to a saturable receptor-dependent binding and uptake of EGF-Gen, that has been reported to occur with unconjugated human EGF in rats at a dose level of  $100 \mu\text{g}/\text{kg}$  (27). As a result of the increase in AUC, the clearance (i.e., Dose/AUC) of EGF-Gen significantly decreased with this dose escalation (Table 4, Figure 12A). The dose-dependent decrease in clearance was not associated with significant differences in  $t_{1/2\beta}$  values [ $1.3 \pm 0.2$  vs  $2.1 \pm 0.3$  for non-tumor bearing mice, and  $1.6 \pm 0.4$  vs  $1.0 \pm 0.1$  for tumor bearing mice], which is in accord with the published observations of Kim et al. (26, 27). These results taken together with previous reports regarding the pharmacokinetics of unconjugated EGF are consistent with the notion that the initial redistribution of EGF-Gen from plasma to EGF-R<sup>+</sup> cells in various tissues determining the  $t_{1/2\alpha}$  values is affected by factors influencing the binding of EGF-Gen to EGF-R<sup>+</sup> cells (e.g. affinity of the EGF-Gen conjugate for EGF-R, number of EGF-R<sup>+</sup> targets in the extravascular compartments), while the later phase of removal from plasma determining the  $t_{1/2\beta}$  values is likely affected by the EGF-R turnover rates and dose-independent disassociation of EGF-Gen from surface EGF-R molecules.

**Pharmacodynamic Features and Toxicity of EGF-Gen in Cynomolgus Monkeys.** Because EGF-Gen was not toxic to healthy mice even at doses as high as  $40 \text{ mg/kg}$  given as a single dose or  $140 \text{ mg/kg}$  given in multiple doses despite the cross-reactivity of human EGF with murine EGF-R, we postulated that such systemic exposure levels could also be achieved in cynomolgus monkeys without excessive toxicity. To test this hypothesis, we measured in cynomolgus monkeys the systemic exposure levels achieved after treatment with  $50 \mu\text{g}/\text{kg}/\text{day} \times 10$  days and  $100 \mu\text{g}/\text{kg}/\text{day} \times 10$  days. The plasma concentration-time curves of EGF-Gen in monkeys were also biphasic (Figure 12B). The volume of

distribution and clearance tended to decrease as the daily dose increased from 50 µg/kg to 100 µg/kg, similar to what was observed in mice. As shown in Table 4, treatment with 100 µg/kg/day EGF-Gen yielded an AUC of 1400 µg\*hr/L. This systemic exposure level is much higher than the target AUC of 16 µg\*hr/L, which was found to be effective in the SCID mouse model of human breast cancer.

Notably, no clinical or laboratory evidence of significant toxicity was observed in these monkeys, except for a transient alopecia in two of the monkeys (Table 5). In particular, we observed no gastrointestinal or hepatic toxicity. No histopathologic lesions were found in the organs of EGF-Gen treated monkeys that were electively euthanized. Thus, EGF-Gen concentrations higher than those which are required to elicit therapeutic efficacy against human breast cancer cells in the SCID mouse xenograft model of human breast cancer were achieved in cynomolgus monkeys without significant systemic toxicity.

We also examined the anti-cancer activity of plasma samples from EGF-Gen-treated monkeys by determining their ability to inhibit the in vitro clonogenic growth of the human breast cancer cell lines MDA-MB-231 and BT-20. As detailed in Table 6, 1:50 PBS-diluted plasma samples obtained at 1 hour after treatment with 1 mg/kg EGF-Gen (but not 1:50 diluted pretreatment plasma samples from the same monkeys) abrogated the in vitro colony formation by these breast cancer cell lines. Notably, excess unconjugated EGF (but not excess G-CSF) could competitively block the cytotoxicity of the EGF-Gen-containing monkey plasma samples (Table 6). These results confirmed the biologic activity and stability as well as EGF-R-specificity of the circulating EGF-Gen molecules in cynomolgus monkeys.

Protein tyrosine kinases have long been suspected to play pivotal roles in regulation of cell survival in cancer cells (19, 28-37). Our recent studies provided experimental evidence that the EGF-R-associated PTK complexes are of vital importance for the survival of breast cancer cells and therefore EGF-R may serve as a suitable target for biotherapy of breast cancer using PTK inhibitors (18). EGF-Gen is an experimental anti-cancer drug which targets the naturally occurring PTK inhibitory isoflavone Gen to the membrane-associated anti-apoptotic EGF-R/PTK complexes and triggers apoptotic cell death (18). In the present study, we examined the in vivo anti-cancer activity, pharmacokinetic features, as well as toxicity

**Table 5. Toxicity of EGF-Genistein in Cynomolgus Monkeys**

System	Grade of Maximum Toxicity (Time)				
	52 B 0.025 mg/kg/day x 10d	52 A 0.050 mg/kg/d x 10d	52 I 0.100 mg/kg/d x 10d	63 A 0.100 mg/kg/d x 10d	63 F 0.1 mg/kg/d x 10d
Activity/Feeding	0	2 (d7-9)	2 (d5-8)	1	0
Fever	0	0	0	0	0
Weight loss	0	0	2 (d12)	0	0
Skin (Alopecia)	0	2 (d13-50)	1	0	0
Cardiac					
Tachycardia	1	1	1	2 (d8)	1
Hypotension	0	0	0	0	0
Pulmonary					
Clinical	0	0	2 (d10)	0	0
Respiratory rate	0	2 (d3-7)	0	0	0
Renal					
Creatinine	0	0	0	0	1
Electrolytes	0	0	0	0	0
Proteinuria	0	1	0	1	0
Hematuria	0	0	0	0	0
Liver					
ALT	2 (d14-18)	2 (d2-3)	1	1	1
Bili	0	0	0	0	0
Gastrointestinal					
Nausea/Vomiting	0	0	0	0	0
Diarrhea	1	0	1	0	0
Constipation	0	0	0	0	0
Nervous System					
Central	0	0	0	0	0
Peripheral	0	0	0	0	0
Coagulation					
PT	0	0	1	0	0
PTT	0	0	0	0	0
Infection	0	0	1	0	0
Blood					
Neutropenia	0	0	0	0	0
Anemia	2 (d7-10)	2 (d9-10)	1	2 (d5-6)	1
Thrombocytopenia	0	0	0	0	0
Metabolic	0	0	1	0	1

Cynomolgus monkeys were treated with intravenous infusions of EFG-Gen or Gen, as described in the Methods. For each >Grade I toxicity the onset and duration of toxicity are indicated in parentheses. ALT, alanine aminotransferase; Bili, bilirubin. Monkeys were electively euthanized at the following time points after initiation of therapy: 52 B, 182days; 52 A 186days; 52 I, 127days; 63 A, 15days; 63 F, 30days; 52 F, 135days; 52 G, 135days. No test substance related histopathologic lesions were found in any of the monkeys.

**Table 6. In Vitro Anti-tumor Activity of Plasma from EGF-Genistein-treated Cynomolgus Monkeys Against EGF-R<sup>+</sup> MDA-MB231 Human Breast Cancer Cell Lines**

Plasma Samples	Mean No. Colonies/10 <sup>5</sup> Cells	% Inhibition
None	348.5 (344, 353)	—
<b>Monkey 52A (EGF-Gen Dose = 50 µg/kg)</b>		
Pretreatment	354.5 (321, 388)	0
1 hour posttreatment	28.5 (21, 36)	91.8
1 hour posttreatment + 10 µg/ml EGF	301.5 (289, 314)	13.5
1 hour posttreatment + 10 µg/ml G-CSF	21.5 (16, 27)	93.8
<b>Monkey 52I (EGF-Gen Dose = 100 µg/kg)</b>		
Pretreatment	323.5 (320, 327)	7.2
1 hour posttreatment	0 (0, 0)	>99.7
1 hour posttreatment + 10 µg/ml EGF	293.0 (278, 308)	15.9
1 hour posttreatment + 10 µg/ml G-CSF	0 (0, 0)	>99.7
<b>Monkey 63A (EGF-Gen Dose = 100 µg/kg)</b>		
Pretreatment	395.0 (391, 399)	0
1 hour posttreatment	4.5 (2, 7)	98.7
1 hour posttreatment + 10 µg/ml EGF	279.5 (276, 283)	19.8
1 hour posttreatment + 10 µg/ml G-CSF	2.5 (1, 4)	99.3
<b>Monkey 63F (EGF-Gen Dose = 100 µg/kg)</b>		
Pretreatment	323 (306, 340)	7.3
1 hour posttreatment	0 (0, 0)	>99.7
1 hour posttreatment + 10 µg/ml EGF	340 (309, 371)	2.4
1 hour posttreatment + 10 µg/ml G-CSF	0 (0, 0)	>99.7

Cells (10<sup>6</sup>/mL RPMI+10% FCS) were incubated overnight at 37°C with 1:50 (v/v) PBS-diluted plasma samples from EGF-Genistein-treated cynomolgus monkeys. After treatment, cells were washed twice, plated at 10<sup>5</sup> cells/ml in RPMI+10% FCS+ 0.9% methylcellulose in Petri dishes, and cultured for 7 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Subsequently, colonies were enumerated and the % inhibition was calculated using the formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{mean no. colonies in test culture}}{\text{mean no. colonies in control culture}} \times 100.$$

Excess EGF was added to some of the plasma samples to block the activity of EGF-Genistein by competing for the EGF-R molecules on MDA-MB231 cells. Excess G-CSF was used as a control for comparison.

profile of EGF-Gen. Here, we presented experimental evidence that EGF-Gen displays significant anti-tumor activity in a SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period.

The inability of unconjugated Gen or unconjugated EGF plus unconjugated Gen to exhibit significant anti-tumor activity in this SCID mouse model of metastatic human breast cancer demonstrates that the anti-cancer activity of EGF-Gen cannot be attributed to either the EGF or Gen moieties alone. Daily administration of 2 µg EGF-Gen, which contains 309 pmols of Gen in conjugated form, for a total of 10 days was a highly effective treatment regimen, whereas daily administration of 10 µg Gen, which corresponds to 37,000 pmols, alone or in combination with 10 µg unconjugated EGF (5-fold higher dose of EGF than what is contained in 2 µg EGF-Gen) for 10 days was not effective at all. Thus, the conjugation of Gen to the targeting EGF molecule enhanced its *in vivo* activity against breast cancer cells more than 100-fold. These findings confirm and extend our in vitro data demonstrating that, compared to unconjugated Gen, EGF-Gen is >1,000-fold more potent cytotoxic agent against EGF-R<sup>+</sup> human breast cancer cells.

EGF-Gen improved tumor-free survival in a SCID mouse model of human breast cancer at systemic exposure levels non-toxic to mice or cynomolgus monkeys. Therefore, therapeutic levels of EGF-Gen may also be achievable in women with metastatic breast cancer without excessive toxicity. Notably, EGF-Gen was more effective than cyclophosphamide, adriamycin, or methotrexate in our MDA-MB-231 SCID mouse xenograft model of human breast cancer. Furthermore, plasma samples from EGF-Gen treated cynomolgus monkeys elicited potent and EGF-R-specific *in vitro* anti-tumor activity against

EGF-R<sup>+</sup> human breast cancer cell lines. These promising preclinical results obtained with EGF-Gen indicate that further clinical development of this promising new anti-breast cancer agent is warranted. EGF-Gen treatment reduced the growth rate of breast cancer xenografts of 1.0 cm diameter, but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors. This is not surprising especially with the liver having a >40-fold higher partition coefficient than the subcutaneous tumors. Thus, EGF-Gen may be more effective as part of an adjuvant therapy regimen when the disease burden is not very large.

EGF-R overexpression is found in many types of cancer besides breast cancer. Thus, EGF-Gen could potentially be used in several different types of cancer. The EGF-R on cancer cells represents a potential target for other forms of biotherapy as well. Anti-EGF-R antibodies may be useful in the treatment of EGF-R positive malignancies by disrupting EGF-mediated signal transduction events. Whether EGF-Gen is superior to such anti-EGF-R antibodies needs to be examined in appropriate preclinical and clinical settings.

### C. SUMMARY

Epidermal growth factor-receptor (EGF-R)-associated protein tyrosine kinase (PTK) complexes have vital anti-apoptotic functions in human breast cancer cells. We have previously shown that targeting the naturally occurring PTK inhibitor genistein to the EGF-R-associated PTK complexes using the EGF-Genistein (Gen) conjugate triggers rapid apoptotic cell death in human breast cancer cells and abrogates their *in vitro* clonogenic growth. In the present study, we examined the *in vivo* toxicity profile, pharmacokinetics, and anti-cancer activity of EGF-Gen. No toxicities were observed in mice treated with EGF-Gen at dose levels as high as 40 mg/kg administered intraperitoneally (i.p.) as a single dose or 140 mg/kg administered i.p. over 28 consecutive days. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. At 100 µg/kg/d x 10 days (1 mg/kg total dose), which is >100-fold less than the highest tested and nontoxic cumulative dose (i.e., 140 mg/kg) in mice, EGF-Gen was more effective than cyclophosphamide (50 mg/kg/d x 2 days), adriamycin (2.5 mg/kg x 1) or methotrexate (0.5 mg/kg x

1), the most widely used standard chemotherapeutic drugs for breast cancer, and resulted in 60 % long-term tumor-free survival. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period. EGF-Gen treatment reduced the growth rate of breast cancer xenografts of 1.0 cm diameter, but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors. The level of EGF-Gen systemic exposure that was effective in SCID mice was achieved in cynomolgus monkeys without any significant side effects detectable by clinical observation, laboratory studies, or histopathological examination of multiple organs. EGF-Gen might be useful in treatment of breast cancer as well as other EGF-R positive malignancies.

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### III. CONCLUSIONS

EGF-Gen conjugate inactivates the EGF-R tyrosine kinase as well as ErbB2, ErbB3, and Src protooncogene family PTK in breast cancer cells triggering apoptosis and clonogenic cell death. Our results indicate that the EGF-R-associated PTK complexes have vital anti-apoptotic functions in human breast cancer cells and may therefore be used as therapeutic targets.

In apoptosis assays, 10 $\mu$ g/ml (=37 $\mu$ M) Gen was not active against MDA-MB-231 and BT-20 breast cancer cells, whereas 1 $\mu$ g/ml (0.137  $\mu$ M) EGF-Gen, which contains 270-fold less Gen was active. In clonogenic assays, the IC<sub>50</sub> values for EGF-Gen against MDA-MB-231 and BT-20 breast cancer cells were >1,000-fold lower than those of unconjugated Gen (30 nM vs 112-119  $\mu$ M). Thus, the conjugation of Gen to the targeting EGF molecule substantially enhances its cytotoxic activity against human breast cancer cells. This may in part be due to the delivery of more Gen molecules to cancer cells, thereby increasing the intracellular Gen concentration, by this targeted biotherapy approach. We further postulate that the binding of EGF-Genistein to the EGF-R brings Gen in direct contact with EGF-R tyrosine kinase as well as Src family PTK associated with the EGF-R. The inhibitor is held in close proximity to the EGF-R and associated PTK because of its covalent attachment to EGF. Localization of the Gen molecule in close proximity to the ATP-binding domains of the EGF-R associated PTK may increase the effective binding constant by both reducing entropy and providing additional linker binding contacts and lead to sustained inhibition of the PTK. Decreasing the effective off-rate of Gen by conjugating it to EGF may also promote covalent modification of the EGF-R-associated PTK by covalent modification, reminiscent of the oxidative inactivation of CD19-associated Src family PTK by B43-Gen, an anti-CD19 antibody-Gen immunoconjugate.

EGF-Gen displays significant anti-tumor activity in a SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter

with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period.

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## **V. APPENDICES**

### **publications**

## **DOCUMENT 2. Year 1998 Annual Report**

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## **INTRODUCTION**

We have continued our efforts to optimize the design of the EGF-Genistein and related tyrosine kinase inhibitor conjugates. The goal of these efforts is to prepare a new generation of EGF conjugates with unprecedented activity as well as stability. The design optimization represents work done at the Hughes Institute whereas the mouse and monkey studies are being conducted at the University of Minnesota. The work as well as analyses are ongoing and no conclusions are yet possible as to whether or not the novel EGF conjugates will be superior to the first generation EGF conjugates. Depending on these results, we will pick the most promising conjugate and start its use as part of combined biochemotherapy regimens, as originally proposed in our application.

## **BODY**

### **SECTION I: DESIGN OPTIMIZATION**

### **MATERIALS AND METHODS**

**Preparation of EGF-Genistein and Related Conjugates .** rhEGF was produced in *E. coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies and the mature protein was recovered by trypsin-cleavage followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of  $4.6 \pm 0.2$ . The endotoxin level was 0.17 EU/mg.

The recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) was initially employed in the synthesis of the EGF-Genistein(Gen) conjugates. Sulfo-SANPAH was dissolved in DMSO and used to modify EGF at molar ratios of 1:1 - 1:10, EGF to crosslinker. Following size-exclusion chromatography to remove unreacted crosslinker and small molecular weight reaction products, the modified rhEGF was mixed with a 10:1 or 20:1 molar ratio of Gen (LC Laboratories, Woburn, MA) [50 mM solution in dimethyl sulfoxide (DMSO)] and then irradiated for 10 - 15 min with long-wave UV light ( 366 nm Model UVGL-58 Mineralight; UVP, Upland, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a molar excess of Genistein resulted in the attachment of Gen to lysine 28, lysine 48, or the N-terminal residue of EGF. Excess Gen in the reaction mixture was removed by passage through a G25-Sephadex pre-packed column, and 12 kDa EGF-EGF homoconjugates with or without conjugated Gen, as well as higher molecular weight reaction products, were removed by size-exclusion high-performance liquid chromatography (HPLC, Beckman System Gold).

In addition to Sulfo-SANPAH, we also used the following crosslinking agents obtained from Pierce Chemical Company: N-5-azido-2-nitrobenzoyloxysuccinimide(ANB-NOS), Sulfosuccinimidyl 2-[m-azido-o-nitrobenzamido]ethyl-1,3'-dithiopropionate(SAND), and Sulfosuccinimidyl(perfluoroazidobenzamido)ethyl-1,3-dithiopropionate(SFAD). These crosslinkers are of different chain lengths, ANB-NOS being the shortest at 7.7 Å, and all have a phenyl azide at one end to react with Genistein following photolysis. The other end of the crosslinker contains an N-hydroxysuccinimide ester to react with protein amino groups. SAND and SFAD are cleavable by thiols. We have also used p-

azidophenylglyoxal monohydrate(APG)(9.3 Å) as an arginine and photoreactive crosslinking agent.

To avoid exposing EGF to the possible harmful effects of UV light, we have also photolyzed the crosslinker-Genistein mixture prior to the addition of EGF. We dissolved both the crosslinker and Genistein in DMSO and mixed them together using a 20:1, 10:1, or 2.5:1 molar ratio of Genistein to crosslinker. Photolysis was performed at room temperature for periods of time from 15 - 60 minutes using either a Model UVM-57(302 nm mid-range wavelength) or Model UVGL-58(366 nm longwave) UV lamp from UVP(Upland, CA). Following photolysis, the mixture was added to a solution of EGF(in PBS) at a molar ratio of 10:1, crosslinker:EGF in a maximum final DMSO concentration of 10%.

In an effort to generate more potent EGF conjugates, we have also used two Genistein analogues, DDE24 and DDE41, which have themselves been shown to possess cytotoxic activity in in vitro systems. These compounds have been modified to contain an N-hydroxysuccinimide ester for direct conjugation to EGF in the absence of photolysis.

**HPLC Analysis.** Reverse phase HPLC using a Hewlett-Packard (HP) 1100 series HPLC instrument was used to monitor and characterize the EGF-Gen conjugation. Analytical HPLC was performed using a Spherisorb ODS-2 reverse phase column (250x4 mm, Hewlett-Packard). Prior to the HPLC runs, a Beckman DU 640B spectrophotometer was used to generate a UV spectrum for each of the samples to ascertain the  $\lambda_{max}$  for EGF-Gen, modified and unmodified EGF. Each HPLC chromatogram was subsequently run at wavelengths of 220, 280, and 480 or 308 nm using the multiple wavelength detector option supplied with the instrument to ensure optimal detection of the individual peaks in the chromatogram. Five - 100

$\mu$ L samples were applied to the above column and analysis was achieved using a gradient flow consisting of 20% to 100% eluent D in a time interval of 0 to 50 min. Eluent A consisted of a mixture of 0.1% trifluoroacetic acid(TFA) in water and eluent D contained 80% acetonitrile (CH<sub>3</sub>CN), 20% H<sub>2</sub>O, and 0.1% TFA.

Size-exclusion chromatography was carried out using a Beckman System Gold Instrument equipped with a TSKG3000SW column. The column was equilibrated in 100 mM sodium phosphate buffer, pH 6.8 at a flow rate of 3 mL/minute.

**Mass Spectrometry.** Mass spectrometric analysis was routinely performed to determine the relative molecular weights of the modified EGF and EGF-Genistein conjugates. A Hewlett-Packard Model G2025A matrix-assisted laser desorption/ionization mass spectrometer with linear time-of-flight mode (MALDI-TOF). In conjunction with the Hewlett-Packard instrument were a sample preparation assembly model G2024A including a high vacuum pump and a Dos-Chem station controller model G1030A. Before starting the experiment, the instrument was calibrated with protein standards G2025A supplied by Hewlett-Packard; mass calibration was used by peak centroiding at the 80% level. Sinnapinic acid(Hewlett-Packard) was used as a matrix source. Samples were prepared by spotting 1  $\mu$ L of a mixture of protein, in phosphate buffer, with the matrix solution(1:1, v/v) on the gold surface of the probe with subsequent evaporation under vacuum. Ionization was accomplished with a laser radiating at a 337-nm wavelength(5 ns pulses, laser energy 1.97 uJ) in both single shot and multiple shot modes. The analyzer was used in the linear mode at an accelerating voltage of 28 kV. The obtained spectra represent the sum of consecutive laser shots and have not been smoothed.

**SDS-PAGE Analysis.** SDS-PAGE was used to monitor the preparation and purification of the EGF-Genistein conjugates. 15% tricine running gels were stained with Coomassie Blue to visualize the protein bands.

**Breast Cancer Cells.** MDA-MB-231 (ATCC HTB-26) is an EGF-R positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51 year old patient. BT-20 (ATCC HTB-19) is another EGF-R positive breast cancer cell line isolated from the primary breast tumor of a 74 year old patient with grade II mammary adenocarcinoma. MDA-MB-231 cells are cultured in Leibovitz's L-15 medium plus glutamine; BT-20 breast cancer cells are maintained in MEM medium containing 0.1 mM NEAA and Earle's BSS. Both media are further supplemented with 10 % fetal bovine serum. For subculturing, medium is removed from the flasks containing a confluent layer of cells and fresh 0.25% trypsin added for 1-2 min. Trypsin is removed and cultures incubated for 5-10 min at 37°C until the cells detached. Fresh medium is then added and the cells aspirated and dispensed into new flasks.

**Cytotoxic Activity of EGF-Genistein and Related EGFCconjugates.** The specific cytotoxic activity of the EGF-Genistein conjugates is determined initially using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim Corp., Indianapolis, IN). Briefly, exponentially growing breast cancer cells are seeded into a 96-well plate at a density of  $2.0 \times 10^4$  cells/well and incubated for 24 hr at 37°C prior to drug exposure. On the day of treatment, culture medium is carefully aspirated from the wells and replaced with fresh medium containing the EGF-Genistein conjugates or unconjugated EGF. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 48 - 72 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere(BT-20 cells; MDA-MB-231 cells are incubated in the absence of CO<sub>2</sub>). To each well, 10 µl of MTT (0.5 mg/ml final concentration) was

added and the plates incubated at 37°C for 4 hours to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized for a minimum of 4 hr at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbance of each well is measured in a microplate reader (Labsystems) at 540 nm. The absorbance is a measure of cell viability; the greater the absorbance the greater the cell viability.

**Colony Assays.** After overnight treatment with EGF-Gen or PBS, cells were resuspended in clonogenic medium consisting of alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/mL/dish and cultured in a humidified 5% CO<sub>2</sub> incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as % inhibition of clonogenic cells at a particular concentration of the test agent using the formula: % Inhibition = (1 - Mean # of colonies [Test] / Mean # of colonies [Control]) x 100.

## RESULTS AND DISCUSSION

Our initial EGF-Genistein conjugates were formed using Sulfo-SANPAH as the photolabile crosslinker. We used MALDI-TOF mass spectrometry as a means of monitoring the modification of EGF using different molar ratios of crosslinker to EGF. **Figure 1** shows an example of these results which indicate that very little unmodified EGF (mass of 6200 daltons) remained when 7.5:1 or 10:1 molar ratios were used. In subsequent experiments EGF was modified using a 10:1 molar ratio of Sulfo-SANPAH followed by photolysis in the presence of longwave UV and a 10 - 20-fold molar excess of Genistein. Size-exclusion HPLC revealed the presence of high-molecular weight material and SDS-

PAGE showed the presence of EGF multimers (**Figure 2**).

We also noted that this EGF conjugate precipitated out of solution during short-term storage at 4° C or when frozen for longer periods of time further reducing the yield of the active EGF - Gen conjugate.

Photolyzing the SANPAH-modified EGF at high protein concentrations appeared to be causing the formation of EGF-EGF multimers and denaturing the EGF so we carried out photolysis on the Sulfo-SANPAH-Genistein mixture(in DMSO) prior to the addition of the EGF. This "pre-photolysis" mixture contained a 20:1 molar excess of Genistein to increase the opportunity for the active nitrene to link to Genistein rather than to another SANPAH or EGF molecule. EGF was added to this mixture following photolysis and unreacted SANPAH and Genistein were removed using G-25 Sephadex column chromatography. Size-exclusion HPLC analysis revealed the presence of high molecular weight aggregates. eluting from 35 - 45 minutes post-injection (**Figure 3 B**). Unmodified EGF typically elutes in this system at 58 - 62 minutes (**Figure 3A**). We observed less aggregation if a 2:1 instead of a 4:1 molar ratio of pre-photolyzed SANPAH - Genistein is used to modify EGF(**Figure 3C**),

We then substituted shorter chain-length and less hydrophobic crosslinkers for SANPAH in order to reduce aggregation due to protein-protein hydrophobic interactions. The short-chain crosslinker, ANB-NOS, results in less precipitation/aggregation than was seen using Sulfo-SANPAH. Since Genistein is relatively insoluble in aqueous solutions, we carried out the pre-photolysis using a 2.5:1 or 10:1 molar ratio of Genistein to crosslinker and a 10:1 ratio of crosslinker-Genistein to EGF. The final DMSO concentration was maintained at 10%.

**Figure 4A,B** shows an initial size-exclusion HPLC purification of EGF-ANB-NOS-Gen conjugates prepared using the above ratios and 15

minutes of longwave UV photolysis. Less aggregation has occurred when the 10:1 ratio is compared to the 2.5:1 ratio and both are significantly less when the ANB-NOS-Gen mixture is pre-photolyzed than when ANB-NOS-modified EGF is mixed with Genistein and then exposed to UV(**Figure 4C**). The SDS-PAGE gel shown in **Figure 5** also indicates that only small amounts of EGF multimers are formed under these conjugation conditions and that size-exclusion HPLC can be used to remove the aggregates.

All of the EGF-ANB-NOS-Gen conjugates possessed some activity in the MTT assay when tested against the BT-20 and/or MDA-MB231 breast cancer cell lines(**Figure 4**). The HPLC-purified 10:1, 10:1 pre-photolyzed conjugate was the most active exhibiting maximum inhibition at a concentration of less than 1 ug/mL.

**Figure 6** shows size-exclusion HPLC profiles that were obtained for EGF conjugates prepared using 10:1 ratios of the Genistein analogs, DDE24 and DDE41. These compounds contain an NHS ester and were directly linked to EGF in PBS buffer without photolysis. The EGF-DDE41 conjugate(A) appeared to contain more aggregated protein than the EGF-DDE24 conjugate(B). The HPLC-semipurified EGF-DDE41 conjugate did appear to have some inhibitory activity in the MTT assay (**Figure 6**).

## **SECTION II. ANIMAL STUDIES**

### **MATERIALS AND METHODS**

The detailed procedures for murine and primate toxicity studies were detailed in the original grant application and also reported in the previously submitted manuscripts regarding the animal toxicity of the first generation EGF conjugates.

### **RESULTS AND DISCUSSION**

We have examined the toxicity of EGF SANPAH conjugates of DDE-24 and DDE-41 as well as EGF ANB-NOS conjugates of Genistein, DDE-24, and DDE-41. As shown in **Figure 7**, no toxicity and no fatalities were observed with any of these treatments. A detailed report of the histopathological study is enclosed as **Appendix 1**. No test article related histologic lesions were found in any of the mice treated with our new generation EGF conjugates.

We have next examined the toxicity of EGF-ANB-NOS-Genistein and EGF-ANB-NOS-DDE41 (EGF-41) in cynomolgus monkeys. Both agents were well tolerated by monkeys. A detailed report of the clinical findings and raw data is enclosed as **Appendix 2**. The monkeys treated with EGF-41 have been sacrificed and the monkeys treated with EGF-ANB-NOS-Genistein will be sacrificed on October 13, 1998 and a detailed histopathology report will be submitted after the analysis of the tissues. The blood samples collected for pharmacokinetic studies have been frozen for future analysis.

### **Figure Legends**

**Figure 1** - Figure 1 shows the results of MALDI-TOF mass spectrometry of EGF and modified EGF preparations. The relative abundance of various molecular species are indicated for unmodified EGF, EGF modified with 1:1 - 1:10 molar ratios of Sulfo-SANPAH, and EGF modified with a 1:10 ratio of ANB-NOS.

**Figure 2** - Figure 2 is a 15% tricine SDS-PAGE running gel stained with Coomassie Blue to show unmodified EGF and a partially purified EGF-Genistein conjugate prepared by photolyzing SANPAH-modified EGF in the presence of Genistein. Multimers of EGF can be seen in the lanes containing higher amounts of EGF-Genistein .

**Figure 3** - Figure 3 shows examples of size-exclusion HPLC profiles of unmodified EGF(A), an EGF-Genistein conjugate(prepared using a 1:4 ratio(B) and a 1:2 ratio(C) of EGF to SANPAH and a pre-photolysis mixture with a 20-fold excess of Genistein to SANPAH). The Beckman System Gold HPLC was equipped with a TSKG3000SW column equilibrated in 100 mM sodium phosphate buffer, pH 6.8, at a flow rate of 3 mL/minute.

**Figure 4** - Figure 4 shows HPLC patterns of EGF-Genistein conjugates prepared using the ANB-NOS crosslinker at a 1:10 ratio of EGF to crosslinker and a 2.5:1 ratio(A) or a 10:1 ratio(B) of Genistein to ANB-NOS in the pre-photolysis mixture. Figure 4C shows the HPLC pattern for an EGF-ANB-NOS-Gen conjugate prepared by photolyzing the modified EGF in the presence of a 20-fold excess of Genistein. Results of MTT assays are also presented for the various EGF-Genistein conjugates.

**Figure 5** - Figure 5 shows a 15% tricine SDS-PAGE running gel stained with Coomassie Blue to show the initial size-exclusion HPLC purification of an EGF-ANB-NOS-Gen conjugate prepared using 10:1 ratios of Genistein to

ANB-NOS( the pre-photolysis mixture was then irradiated with longwave UV for 60 minutes at room temperature)and ANB-NOS to EGF.

**Figure 6** - Figure 6 shows size-exclusion HPLC profiles of the EGF-DDE41(A) and EGF-DDE24(B) conjugates. High molecular weight aggregates are seen eluting from 34 - 44 minutes in the EGF-DDE41 pattern while the EGF-DDE24 preparation has very little of this material. MTT assay results are included for the HPLC-semi-purified EGF-DDE41 conjugate.

**Figure 7** - These figures show the proportion of mice alive after treatment with the various EGF conjugates. The 100% survival observed for each treatment protocol demonstrates that none of these new generation EGF conjugates are toxic to mice.

# **Appendix I**

**Histopathologic Evaluation of Tissues from BALB/C Mice on an  
Intraperitoneal Toxicity Study of EGF-Conjugates:**

**EGF/24  
EGF/41  
EGF/ANB-NOS-24  
EGF/ANB-NOS-41  
EGF/ANB-NOS-GEN**

**Experiment Dates:  
8/4/98**



Date: 10/9/98

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**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

**A. MATERIAL AND METHODS:**

1. The study was performed as follows:

a. **EGF-Conjugates:**

1. **EGF/24:**

- a. **GROUP 1:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/24, 100 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).
- b. **GROUP 2:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/24, 200 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).
- c. **GROUP 3:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/24, 400 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).
- d. **GROUP 4:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/24, 800 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).

2. **EGF/41:**

- a. **GROUP 5:** On 8/4/98, 2 female BALB/C mice received a single IP (intravenous) injection of EGF/41, 100 µg, in 200 µl PBS (phosphate buffered saline). Both mice were euthanized clinically healthy on day 30 (9/3/98).
- b. **GROUP 6:** On 8/4/98, 2 female BALB/C mice received a single IP (intravenous) injection of EGF/41, 200 µg, in 200 µl PBS (phosphate buffered saline). Both mice were euthanized clinically healthy on day 30 (9/3/98).

**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

**3. EGF/ANB-NOS-24:**

- a. **GROUP 7:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-24, 100 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).
- b. **GROUP 8:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-24, 200 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).

**4. EGF/ANB-NOS-41:**

- a. **GROUP 9:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-41, 100 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).
- b. **GROUP 10:** On 8/4/98, 2 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-41, 200 µg, in 200 µl PBS (phosphate buffered saline). Both mice were euthanized clinically healthy on day 30 (9/3/98).

**5. EGF/ANB-NOS-GEN:**

- a. **GROUP 11:** On 8/4/98, 2 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-GEN, 100 µg, in 200 µl PBS (phosphate buffered saline). Both mice were euthanized clinically healthy on day 30 (9/3/98).
- b. **GROUP 12:** On 8/4/98, 2 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-GEN, 200 µg, in 200 µl PBS (phosphate buffered saline). Both mice were euthanized clinically healthy on day 30 (9/3/98).
- c. **GROUP 13:** On 8/4/98, 3 female BALB/C mice received a single IP (intravenous) injection of EGF/ANB-NOS-GEN, 800 µg, in 200 µl PBS (phosphate buffered saline). All 3 mice were euthanized clinically healthy on day 30 (9/3/98).

**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

b. **PBS Treatment (Control Group):**

1. **GROUP 14:** On 8/4/98, 6 female BALB/C mice received a single IP (intraperitoneal) injection of 200 $\mu$ l PBS (phosphate buffered saline). All 6 mice were euthanized clinically healthy on day 30 (9/3/98).
- c. At necropsy, no gross lesions were observed in any group.

2. **Clinical Phase, Necropsy and harvesting of tissues:**

- a. The clinical phase, necropsy and harvesting of tissues was performed at the Wayne Hughes Institute Pre-Clinical Laboratory, 2680 Patton Road, Roseville, MN 55113.
- b. At death, all mice had routine postmortem examinations. All tissues were collected, fixed in 10% formalin, and processed for histologic sectioning in a routine manner.
- c. The histology slides were stained with Hematoxylin and Eosin.
- d. The histologic evaluation of the tissues and report compilation was done by B.Waurzyniak, DVM., MS., (veterinary pathologist).

**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

**B. RESULTS:**

1. **TABLE 1: Treatment Table and Mouse Identification. .... 8/4/98**

TREATMENT:	EGF/24				EGF/41		EGF/ANB-NOS-24		EGF/ANB-NOS-41		EGF/ANB-NOS-GEN		PBS	
GROUP:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DOSAGE (µg):	100	200	400	800	100	200	100	200	100	200	100	200	800	0
TX ROUTE:	IP													
Outcome: 1. SM = euthanized moribund 2. SH = euthanized healthy 3. D = died.	All SH.													
Experiment duration (days):	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Mouse ID Numbers:	19095 19096 19097	19102 19103 19104	19064 19100 19101	19061 19062 19063	19070 10979 19078	19077 19078 19078	19091 19092 19093	19090 19098 19099	19060 19073 19074	19071 19072 19074	19075 19076 19076	19143 19144 19142	19140 19141 19142	19094 19085 19086 19087 19088 19089
# Mice / Group	3	3	3	3	2	2	3	3	3	2	2	2	3	6
# Mice Examined	0	0	0	3	0	2	0	3	0	2	0	0	3	6

4. No test agent related lesions were found in any mice in this study.

5. Incidental findings: (see Table 2).

a. Epicarditis, mild, nonsuppurative, focal, chronic was present in:

1. 1/3 (33%) of mice from Group 4 (EGF/24 800 µg);
2. 1/3 (33%) of mice from Group 8 (EGF/ANB-NOS-24 200 µg);
3. 1/6 (17%) of mice from Group 14 (PBS - Control).

b. Epicardial dystrophic mineralization, mild, chronic, was present in:

1. 1/3 (33%) of mice from Group 4 (EGF/24 800 µg);
2. 1/3 (33%) of mice from Group 8 (EGF/ANB-NOS-24 200 µg);
3. 1/3 (33%) of mice from Group 13 (EGF/ANB-NOS-GEN 800 µg);
4. 1/6 (17%) of mice from Group 14 (PBS - Control).

**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

- c. Hepatitis, multifocal, mild, subacute, was present in:
  - 1. 1/3 (33%) of mice from Group 4 (EGF/24 800 µg);
  - 2. 1/2 (50%) of mice from Group 6 (EGF/41 200 µg);
  - 3. 1/6 (17%) of mice from Group 14 (PBS - Control).
- d. Gastritis, mild, focal, non-ulcerative, subacute, was present in:
  - 1. 1/3 (33%) of mice from Group 4 (EGF/24 800 µg);
  - 2. 1/6 (17%) of mice from Group 14 (PBS - Control).
- e. Dystrophic mineralization, of the gastric tunica muscularis, focal, mild, chronic, was present in:
  - 1. 1/3 (33%) of mice from Group 8 (EGF/ANB-NOS-24 200 µg);
  - 2. 1/2 (50%) of mice from Group 10 (EGF/ANB-NOS-41 200 µg);
  - 3. 2/3 (67%) of mice from Group 13 (EGF/ANB-NOS-GEN 800 µg);
  - 4. 1/6 (17%) of mice from Group 14 (PBS - Control).

**C. COMMENTS:**

- 1. The EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN) were non-toxic under the conditions of this study. All mice were euthanized clinically healthy at the end of the study.
- 2. Histologic lesions related to (IP) EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN) were not present in any mice in the study.

**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

**TABLE 2: Histopathologic Evaluation of Tissues from BALB/C Mice on a Toxicity Study of EGF-Conjugates (Experiment Date 8/4/98).**

Group Number:	4	6	8	10	13	14
Treatment:	EGF/24	EGF/41	EGF/ANB-NOS-24	EGF/ANB-NOS-41	EGF/ANB-NOS-GEN	PBS
Treatment Dose (μg):	800	200	200	200	800	0
Total Number of Mice / Group:	3	2	3	2	3	6
Total # of Mice with Histology Examination:	3	2	3	2	3	6
Tissue/Diagnosis/Modifier(S):						
<u>Bone:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Bone Marrow:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Brain:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Heart:</u>						
1. WNL	1	2	1	2	2	4
2. NE	0	0	0	0	0	0
3. Epicarditis, nonsuppurative, mild, focal, chronic	1 (33%)	0	1 (33%)	0	0	1 (17%)
4. Dystrophic mineralization, epicardium, ± fibrosis, mild, focal - multifocal, chronic	1 (33%)	0	1 (33%)	0	1 (33%)	1 (17%)
<u>Kidney:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Large Intestine:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Liver:</u>						
1. WNL	2	1	3	2	3	5
2. NE	0	0	0	0	0	0
3. Hepatitis, multifocal, mixed inflammation, mild, subacute ± focal hepatic necrosis	1 (33%)	1 (50%)	0	0	0	1 (17%)
<u>Lung:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Lymph node:</u>						
1. WNL	3	1	0	1	2	2
2. NE	0	1	3	1	1	4
<u>Ovary:</u>						
1. WNL	1	1	2	1	1	2
2. NE	2	1	1	1	2	4
<u>Pancreas:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0

**Histopathologic Evaluation of Tissues from BALB/C Mice on an Intraperitoneal Toxicity Study of EGF-Conjugates (EGF/24, EGF/41, EGF/ANB-NOS-24, EGF/ANB-NOS-41, and EGF/ANB-NOS-GEN); Experiment Dates 8/4/98.**

**TABLE 4: (Continued).**

Group Number:	4	6	8	10	13	14
Treatment:	EGF/24	EGF/41	EGF/ANB-NOS-24	EGF/ANB-NOS-41	EGF/ANB-NOS-GEN	PBS
Treatment Dose ( $\mu$ g):	800	200	200	200	800	0
<u>Skeletal Muscle:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Skin:</u>						
1. WNL	3	2	3	1	2	6
2. NE	0	0	0	1	0	0
<u>Small Intestine:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Spinal cord:</u>						
1. WNL	0	0	0	0	1	3
2. NE	3	2	3	2	2	3
<u>Spleen:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Stomach:</u>						
1. WNL	2	2	2	1	1	4
2. NE	0	0	0	0	0	0
3. Gastritis, mixed inflammation, mild, focal, non-ulcerative, subacute	1 (33%)	0	0	0	0	1 (17%)
4. Dystrophic mineralization, focal, mild, chronic, tunica muscularis.	0	0	1 (33%)	1 (50%)	2 (67%)	1 (17%)
<u>Thymus:</u>						
1. WNL	3	2	3	2	3	6
2. NE	0	0	0	0	0	0
<u>Urinary Bladder:</u>						
1. WNL	3	1	2	1	2	4
2. NE	0	1	1	1	1	2
<u>Uterus:</u>						
1. WNL	3	2	3	2	2	6
2. NE	0	0	0	0	1	0

WNL = within normal limits. NE = not examined.

% = (number of mice with lesion + total number of mice examined) x 100

## **Appendix II**

### **Monkey 68J EGF/ANB-NOS-Gen Summary**

On 9/29/98, day 1 of this study, Monkey 68J, a female adult cynomologus macaque, was given a 25ml bolus of 5mg EGF/ANB-NOS-Gen intravenously over a two minute time period. A pre infusion pharmacology sample was taken as well as blood for chemistry, CBC, and coagulation tests. A non-sterile urinalysis was also done. Time points for blood draws for the pharmacology were: 10min, 20min, 30min, 1hour, 2hour, 4hour, 8hour, and 12hour. Pharmacology sample were taken every day up to the one week sample and a two week sample was also taken.

Vitals, chemistries, and CBCs were taken on days 1-10 and 15. Coagulation tests were taken on days 1, 4, 7, 10, and 15. A second urinalysis was taken on day 15. Clinical observations are detailed in the attached data forms.

The sacrifice date for this monkey is 10/13/98.

### **Monkey 68N EGF/ANB-NOS-Gen Summary**

On 9/29/98, day 1 of the study, Monkey 68N, a female adult cynomologus macaque weighing 3.9 kg, was given a 25ml bolus of 1mg EGF/ANB-NOS-Gen intravenously over a two minute time period. A pre infusion pharmacology sample was taken as well as blood for chemistry, CBC, and coagulation tests. A non-sterile urinalysis was also done. Time points for blood draws for the pharmacology were: 10min, 20min, 30min, 1hour, 2hour, 4hour, 8hour, and 12hour. Pharmacology samples were taken every day up to the one week sample and a two week sample was also taken.

Vitals, chemistries, and CBCs were taken on days 1-10 and 15. Coagulation tests were taken on days 1, 4, 7, 10, and 15. A second urinalysis was taken on day 15. Clinical observations are detailed in the attached data forms.

Sacrifice date for this monkey is 10/13/98.

# Toxicity of EGF/ANB-NOS-Gen in Cynomolgus Monkeys

System	Grade of Maximum Toxicity (Time)	
	(1 mg, Bolus, IV)	(5 mg, Bolus, IV)
Activity/Feeding	0	0
Fever	0	0
Weight Loss	0	0
Skin (Alopecia)	0	0
Cardiac		
Tachycardia	1	0
Hypertension	NA	NA
Hypotension	NA	NA
Pulmonary		
Clinical	0	0
Respiratory rate	1	0
Renal		
Creatinine	0	0
Electrolytes	0	0
Proteinuria	0	0
Hematuria	0	0

## Toxicity of EGF/ANB-NOS-Gen in Cynomolgus Monkeys

System	Grade of Maximum Toxicity (Time)	
	68N (1 mg, Bolus, IV)	68J (5 mg, Bolus, IV)
Liver		
ALT	1	1 <sup>r</sup>
Bili	0 <sup>r</sup>	
Gastrointestinal		
Nausea/Vomiting	0	0
Diarrhea	0	0
Constipation	0	0
Nervous System		
Central	0	0
Peripheral	0	0
Infection	0	0
Blood		
Leukopenia	0	0
Anemia	2 (d10, 9.5 g/dL*)	2 (d10, 8.6 g/dL*)
Thrombocytopenia	0	0
Metabolic	3 (d1)	2 (d1, 3, 7 - 10)

<sup>r</sup>Bilirubin elevated due to hemolytic sample.

\*Hemoglobin level decreased due to blood draw.

### **Monkey 68I EGF/41 Summary**

On 9/16/98 on day 1 of this study, Monkey 68I, a female adult cynomologus macaque weighing 4.25kg, was given a 25ml bolus of 5mg EGF/41 intravenously during a two minute time period. A pre infusion pharmacology sample was taken as well as blood for chemistry, CBC, and coagulation tests. A non-sterile urinalysis was also done. Time points for blood draws for the pharmacology were: 10min, 20min, 30min, 1hour, 2hour, 4hour, 8hour, and 12hour. Pharmacology samples were also taken every day up to the one week sample and a two week sample was also taken

Vitals, CBCs and chemistries have been taken for days 1-10 and day 15. Coagulation tests were taken on days 1, 4, 7, 10, and 15. Another urinalysis was done on day 15. Clinical observations are detailed on the attached data forms.

This monkey was sacrificed on 9/30/98.

### **Monkey 68K EGF/41 Summary**

On 9/16/98 day 1 of the study, Monkey 68K, an adult female cynomologus macaque, weighing 4.05kg, was given a 25ml bolus of 1mg EGF/41 intravenously over a two minute time period. A pre infusion pharmacology sample was taken as well as blood for chemistry, CBC, and coagulation tests. A non-sterile urinalysis was also done. Time points for blood draws for the pharmacology were: 10min, 20min, 30min, 1hour, 2hour, 4hour, 8hour, and 12hour. Blood was drawn for pharmacology timepoints every day up to the 1 week sample and also a 2 week sample was drawn.

Vitals, chemistries and CBCs were taken on days 1-10 and day 15. Coagulation tests were also taken on days 1, 4, 7, 10, and 15. Clinical observations are detailed on the attached data forms.

This monkey was sacrificed on 9/30/98.

# Toxicity of EGF/41 in Cynomolgus Monkeys

System	Grade of Maximum Toxicity (Time)	
	(1 mg, Bolus, IV)	(5 mg, Bolus, IV)
Activity/Feeding	0	3 (d3)
Fever	0	0
Weight Loss	0	0
Skin (Alopecia)	0	0
Cardiac	0	0
Tachycardia	NA	NA
Hypertension	NA	NA
Hypotension	NA	NA
Pulmonary	0	0
Clinical	0	0
Respiratory rate	0	0
Renal	0	0
Creatinine	0	0
Electrolytes	0	0
Proteinuria	0	0
Hematuria	0	1

## Toxicity of EGF/41 in Cynomolgus Monkeys

System	Grade of Maximum Toxicity (Time)	
	68K (1 mg, Bolus, IV)	68I (5 mg, Bolus, IV)
Liver	0	3 (d15) 0
ALT	0	
Bili	0	
Gastrointestinal	0	
Nausea/Vomiting	0	0
Diarrhea	0	0
Constipation	0	0
Nervous System	0	
Central	0	0
Peripheral	0	0
Infection	0	0
Blood	0	
Leukopenia	0	0
Anemia	0	2 (d10, 8.5 g/dL*)
Thrombocytopenia	0	0
Metabolic	2 (d1, 3, 6, 8 - 10)	3 (d1)

\*Hemoglobin level decreased due to blood draw.

**MONKEY  
TOXICITY AND COMPLICATIONS CRITERIA**

SITE	MEASURE	GRADE				
		WNL	1 (Mild)	2 (Moderate)	3 (Severe)	4(Unaccept)
HEMATOLOGY	1. WBC/ Leukocytosis	4.0 - 14.0 WNL	3.0 - 3.9 14.1 - 20.0	2.0 - 2.9 20.1 - 30.0	1.0 - 1.9 30.1 - 40.0	< 1.0 > 40.0
	2. HgB	> 11.5	10.0 - 11.5	8.0 - 9.9	6.5 - 7.9	< 6.5
	3. PLT	> 150	75.0 - 150.0	50.0 - 74.9	25.0 - 49.9	< 25.0
FEEDING	Feeding Abnormality	none	-----	decreased intake	not eating	dehydration req. IV
GASTROINTEST	Diarrhea	none	mild amt of soft stool	mod amt of soft stool, diarrhea, minimal bleeding, small amt of mucous in stool	watery diarrhea, excessive amt of soft stool, large amt of mucous in stool	bloody diarrhea or severe dehydration due to diarrhea
LIVER	1. Bili	≤ 1.3	1.4 - 1.5	1.6 - 2.0	2.1 - 4.0	> 4.0
	2. ALT	≤ 60	61 - 150	151 - 300	301 - 1200	> 1200
PANCREAS	Amylase	≤ 363	364 - 545	546 - 726	727 - 1815	> 1815
RENAL	1. Urea N	< 20	20 - 39	40 - 59	60 - 79	≥ 80
	2. Creatinine	≤ 1.1	1.2 - 1.5	1.6 - 3.0	3.1 - 6.0	> 6.0
	3. Urine: protein	negative	(1 or more) + 1	(1 or more) + 2 to + 3	(1 or more) + 4	(1 or more) > + 4, marked protein loss
	blood	negative	> 10	see blood	see blood clots	transfusion req. bec of bloody urine
	infection	negative	+ 5 WBC, < 10,000 colonies, (+)	many WBC (++)	sheets of WBC, > 10,000 colonies, (+++) or (++++)	sepsis due to urine dehydr, weight loss, 1.008 - 1.012
	spec. grav.	1.013-1.035	-----	<1.013,>1.035	1.008 - 1.012	-----
PULMONARY	1. Clinical	clear	wheezing	crackle	severe respir distress	-----
	2. Respir Rate: a) conscious	28 - 32	33 - 50	51 - 70	71 - 80	> 80
	b) anesthetized	20 - 32	33 - 50	51 - 70	71 - 80	> 80

SITE	MEASURE	GRADE				
		WNL	1 (Mild)	2 (Moderate)	3 (Severe)	4 (Unaccept)
CARDIAC	1. Murmur	none	slight	significant	very significant	-----
	2. Heart Rate: a) conscious	195 - 265	160 - 195 265 - 300	125 - 159 301 - 335	< 125 > 335	-----
	b) anesthetized	145 - 195	120 - 145 195 - 220	95 - 119 221 - 245	< 95 > 245	-----
	3. Hypertension a) conscious	90 - 130/ 35 - 65	(1-2 readings) 131 - 150/ 66 - 80	(all 3 readings)	> 150/> 80 (req. saline)	-----
	b) anesthetized	45 - 85/ 25 - 45	86 - 105/ 46 - 55		> 105/> 55 (req. saline)	-----
	4. Hypotension a) conscious	90 - 130/ 35 - 65	(1-2 readings) 70 - 89/ 25 - 34	(all 3 readings)	< 70/< 25 (req. saline)	-----
	b) anesthetized	45 - 85/ 25 - 45	25 - 44/ 15 - 24		< 25/< 15 (req. saline)	-----
NEUROLOGY	1. Motor	no change	mild weakness	mod. weakness	severe weakness	paralysis
	2. Examination of Gait	(5) normal strength/ coordination	(4) supportive standing, min. paraparesis/ ataxia	(3) supportive standing, stumbles freq. and falls, mild paraparesis/ ataxia	(2) can't stand, when assisted - stumbles and falls frequently, mod. paraparesis/ ataxia	(1) can't stand, slight movement when held by tail, severe paraparesis
	3. CNS	no change	drowsy	lethargic, very drowsy	seizures	comatose
SKIN	1. Allergic	none	mild rash	swelling, hives, itching	generalized swelling, itching, req. treatment	skin sloughing
	2. Alopecia	none	mild localized loss	complete local loss, mild general loss	severe generalized loss	bald
WEIGHT CHANGE	From 1st day	± 2% - 4.9%	± 5% - 9.9%	± 10 % - 19.9%	≥ 20.0%	-----
COAGULATION	1. INR	< 1.09	1.09 - 1.35	1.36 - 1.59	1.6 - 2.1	≥ 2.2
	2. PTT	< 34.0	34.0 - 54.9	55.0 - 79.5	80.0 - 99.9	≥ 100.0
	3. CFIB (elev = infection)	> 0.15	0.11 - 0.15	0.08 - 0.10	0.05 - 0.07	≤ 0.04

SITE	MEASURE	GRADE				
		WNL	1 (Mild)	2 (Moderate)	3 (Severe)	4(Unaccept)
METABOLIC	1. Anion Gap	≤ 16	17 - 22	23 - 30	31 - 35	≥ 36
	2. Glucose	65 - 115	55 - 64 116 - 160	40 - 54 161 - 250	30 - 39 251 - 500	< 30 > 500
	3. Albumin	≥ 3.5	3.0 - 3.49	2.0 - 2.9	1.5 - 1.9	< 1.5
ACTIVITY	1. Overall Activity Level	no symptoms	symptoms, able to carry out daily activities	minimal prodding required	strong prodding required	can't move even with prodding
	2. Hunched/ Discomfort	none	mild	moderate	mod-severe	severe
TEMPERATURE	Fever/ Hypothermia	97° - 101.5°	101.6° - 103°	103.1° - 104°	> 104°, < 98.5° conc, < 97° anesth (not induced)	consistently > 104°, consistently < 97°
INFECTION		none	runny eyes/nose, cough, mild diarrhea	localized skin infection, severe cold, mod. diarrhea, w/o systemic symptoms	positive culture, w/systemic symptoms	life threatening
OVERALL HEALTH	Not including blood results	-----	mild	moderate	severe	deathly sick

# **Appendix III**

NEW DATA\* [A.03.00]

#5081]

Sample Name EGF  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

1 mg/10 mL

Collected Fri Apr 17 10:45:52 1998  
 Processed Fri Apr 17 10:48:01 1998  
 Printed Fri Apr 17 10:52:41 1998

Sequence Method C:\HPTOFLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 28.5) (50 of 136) Mesa 1 [25-82]  
 Laser Energy 2.28 (0.55) uJ Vacuum 1.44e-006 torr

Mass Range 20000 Da Ion Optics 28.0/7.0 kV

Mass Filter 350 Da Detector -4.75 kV

Data Interval 5.0 nsec Digitizer 1000 mVFS

Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

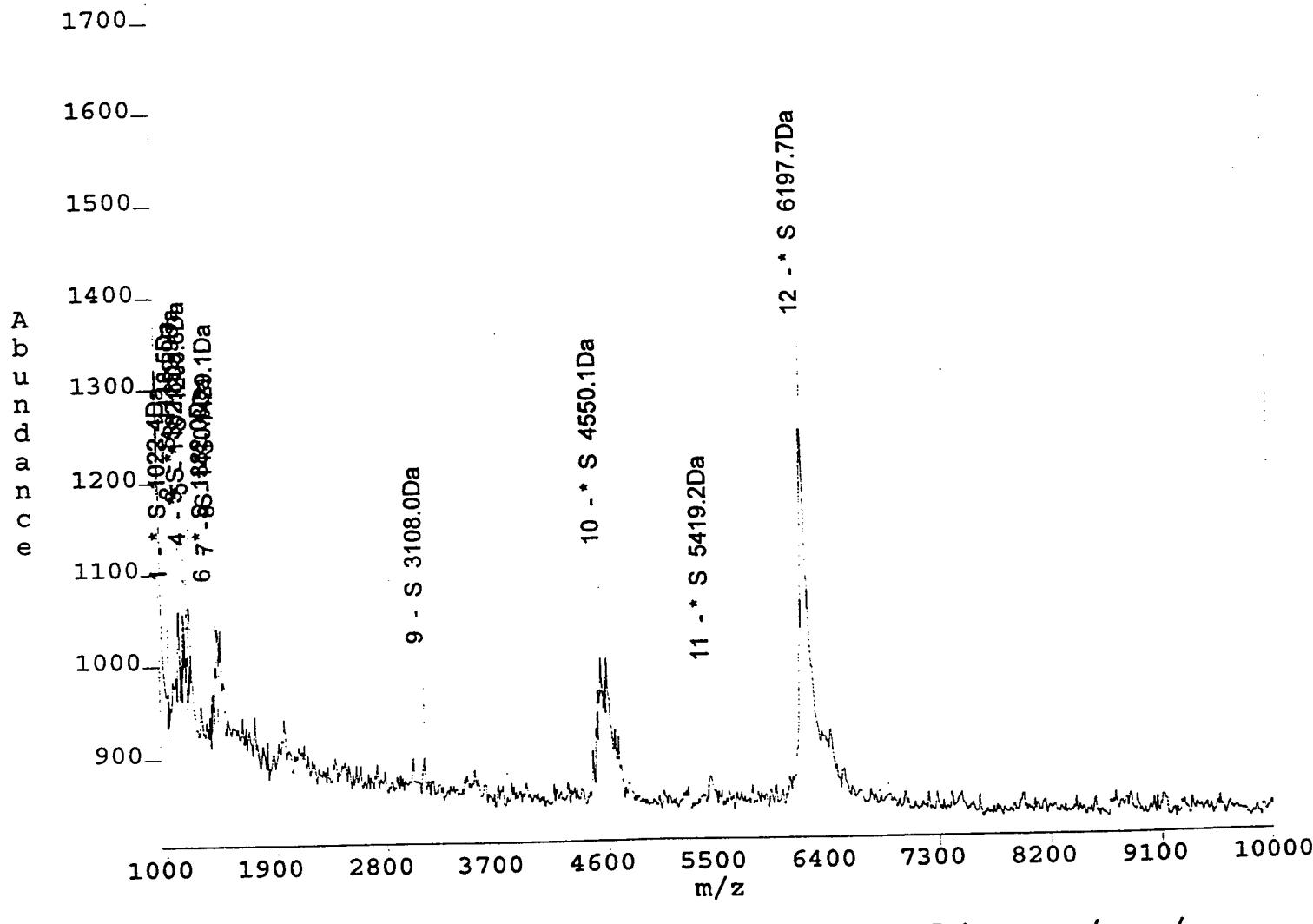
Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

## FIGURE 1A



NEW DATA\* [A.03.00]

#5081]

Sample Name EGF  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

1 mg/10 mL

Collected Fri Apr 17 10:45:52 1998  
 Processed Fri Apr 17 10:48:01 1998  
 Printed Fri Apr 17 10:52:41 1998

Sequence Method C:\HPTOFLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 28.5) (50 of 136) Mesa 1 [25-82]  
 Laser Energy 2.28 (0.55) uJ Vacuum 1.44e-006 torr

Mass Range 20000 Da Ion Optics 28.0/7.0 kV

Mass Filter 350 Da Detector -4.75 kV

Data Interval 5.0 nsec Digitizer 1000 mVFS

Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

Peak	Height	Area	MW	delMW	%err	Name (page 1 of 1)
1 * S	972	903	1022.4			
2 S	1061	1704	1113.5	91.1		
3 * S	1057	1170	1159.9	46.4		
4 * S	1011	892	1182.8	22.9		
5 * S	1065	3920	1205.6	22.7		
6 * S	971	778	1382.0	176.4		
7 S	1000	859	1410.8	28.8		
8 * S	1040	1277	1429.1	18.3		
9 S	899	591	3108.0	1678.9		
10 * S	1003	2400	4550.1	1442.1		
11 * S	873	1005	5419.2	869.0		
12 * S	1246	18962	6197.7	778.5		
13 * S	881	950	12396.7	6199.0		
14 * S	856	604	12546.9	150.2		

\*=Gauss, (D)elected, (C)alibrant/(S)ample, M=Manual, P#=Polymer, ? = changed.

# FIGURE 1A (CONT'D)

NEW DATA\* [A.03.00 #5091]

Sample Name EGF/SANPAH 1:1  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/14/98

Collected Fri Apr 17 11:32:24 1998  
 Processed Fri Apr 17 11:34:03 1998  
 Printed Fri Apr 17 11:34:06 1998

Sequence  
 Method C:\HPTOFOLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 93.0) (50 of 79) Mesa 1 [25-25]  
 Laser Energy 1.23 (0.43) uJ Vacuum 1.80e-006 torr

Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

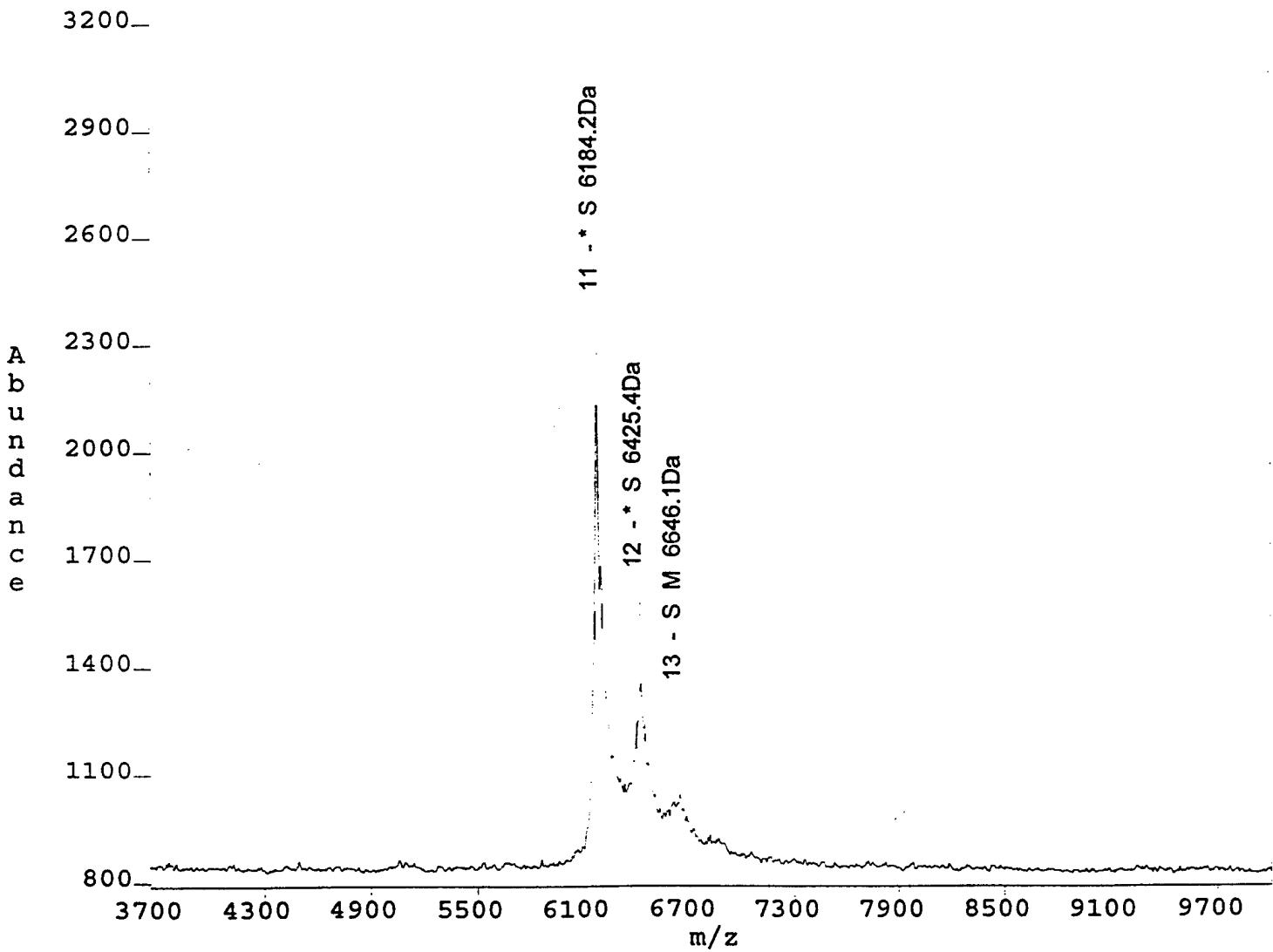
Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

## FIGURE 1B



Sign: \_\_\_\_\_

Date: / /

NEW DATA\* [A.03.00 , #5091]

Sample Name EGF/SANPAH 1:1  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/14/98

Collected Fri Apr 17 11:32:24 1998  
 Processed Fri Apr 17 11:34:03 1998  
 Printed Fri Apr 17 11:34:06 1998

Sequence Method C:\HPTOFOOLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 93.0) (50 of 79) Mesa 1 [25-25]  
 Laser Energy 1.23 (0.43) uJ Vacuum 1.80e-006 torr  
 Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

Peak		Height	Area	MW	delMW	%err	Name (page 1 of 1)
1	*	S	1024	779	977.3		
2	*	S	951	839	997.2	20.0	
3	S		982	582	1136.5	139.2	
4	*	S	1002	1082	1157.7	21.2	
5	*	S	1038	1370	1178.8	21.2	
6	*	S	1089	1889	1201.3	22.4	
7	*	S	935	628	1382.1	180.8	
8	*	S	991	956	1424.4	42.3	
9	*	S	981	786	1446.2	21.8	
10	*	S	922	651	1695.7	249.5	
11	*	S	2131	46211	6184.2	4488.5 - EGF	
12	*	S	1356	5411	6425.4	241.2 - EGF/SAN (1:1)	
13	S	M	1047	2764	6646.1	220.7 - EGF(SAN(1:2?))	
14	*	S	926	2340	12390.9	5744.8	
15	S		1070	687	18094.9	5704.0	

\*=Gauss, (D)elected, (C)alibrant/(S)ample, M=Manual, P#=Polymer, ? = changed.

## FIGURE 1B (CONT'D)

Sign: \_\_\_\_\_

Date: / /

NEW DATA\* [A.03.00 , #5095]

Sample Name EGF/SANPAH 1:5  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/14/98

Collected Fri Apr 17 11:40:46 1998  
 Processed Fri Apr 17 11:42:08 1998  
 Printed Fri Apr 17 11:42:20 1998

Sequence Method C:\HPTOFLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 77.4) (50 of 69) Mesa 5 [25-25]  
 Laser Energy 1.05 (0.17) uJ Vacuum 9.47e-007 torr

Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

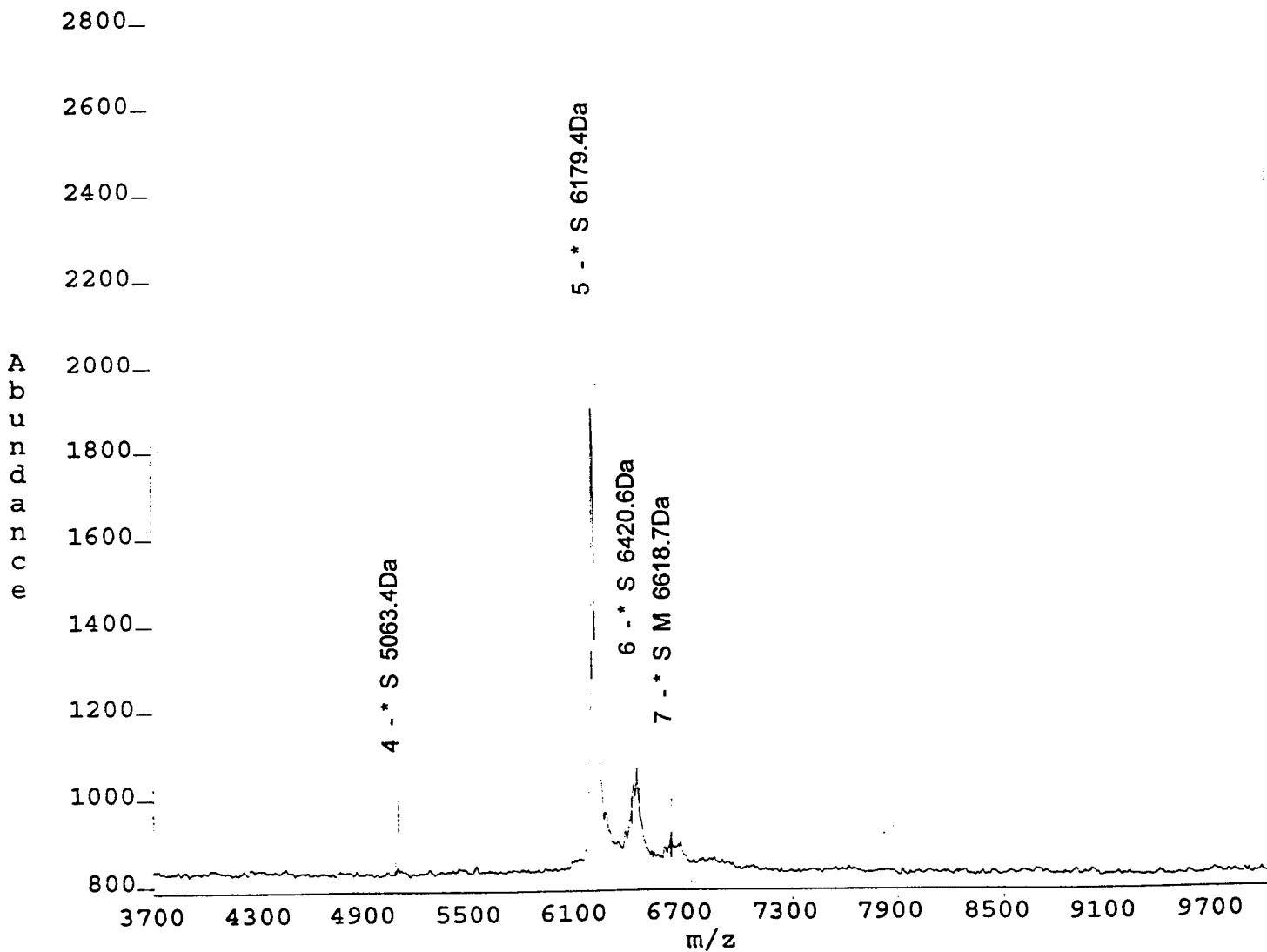
Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

## FIGURE 1C



Sign: \_\_\_\_\_

Date: / /

NEW DATA\* [A.03.00] #5095]

Sample Name EGF/SANPAH 1:5  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/14/98

Collected Fri Apr 17 11:40:46 1998  
 Processed Fri Apr 17 11:42:08 1998  
 Printed Fri Apr 17 11:42:20 1998

Sequence C:\HPTOFLD\METHOD\PEP-NEG.MET  
 Method

Collection Mode Auto Multi Shots (S/N 77.4) (50 of 69) Mesa 5 [25-25]  
 Laser Energy 1.05 (0.17) uJ Vacuum 9.47e-007 torr  
 Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010  
 Calibration - Program Calculated (2-Parameter)  
 Calibration Date Fri Nov 04 15:09:44 1994  
 Calibrator Christopher M. Adams  
 Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

Peak	Height	Area	MW	delMW	%err	Name (page 1 of 1)
1 * S	893	726	1113.2			
2 * S	942	790	1200.5	87.2		
3 * S	891	615	1224.3	23.9		
4 * S	847	510	5063.4	3839.1		
5 * S	1900	20074	6179.4	1116.0		
6 * S	1069	5906	6420.6	241.2		
7 * S M	906	63	6618.7	198.1		
8 * S	891	2289	12363.1	5744.5		
9 S	856	618	12865.1	502.0		

\*=Gauss, (D)elected, (C)alibrant/(S)ample, M=Manual, P#=Polymer, ? = changed.

## FIGURE 1C (CONT'D)

Sign: \_\_\_\_\_

Date: / /

NEW DATA\* [A.03.00] , #5132]

Sample Name EGF/SANPAH 1:7.5  
Preparation PBS  
Matrix Sinnapinic Acid  
User Name L. Ronken  
Department Name Biotherapy  
Application

4/14/98

Collected Mon Apr 20 12:43:25 1998  
Processed Mon Apr 20 12:52:40 1998  
Printed Mon Apr 20 12:54:33 1998

Sequence C:\HPTOFOOLD\METHOD\PEP-NEG.MET\*

Method Collection Mode Single Shots (55 of 263) Mesa 6 [14-117]  
Laser Energy 3.06 (0.52) uJ Vacuum 7.03e-007 torr

Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
Mass Filter 350 Da Detector -4.75 kV  
Data Interval 5.0 nsec Digitizer 1000 mVFS  
Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

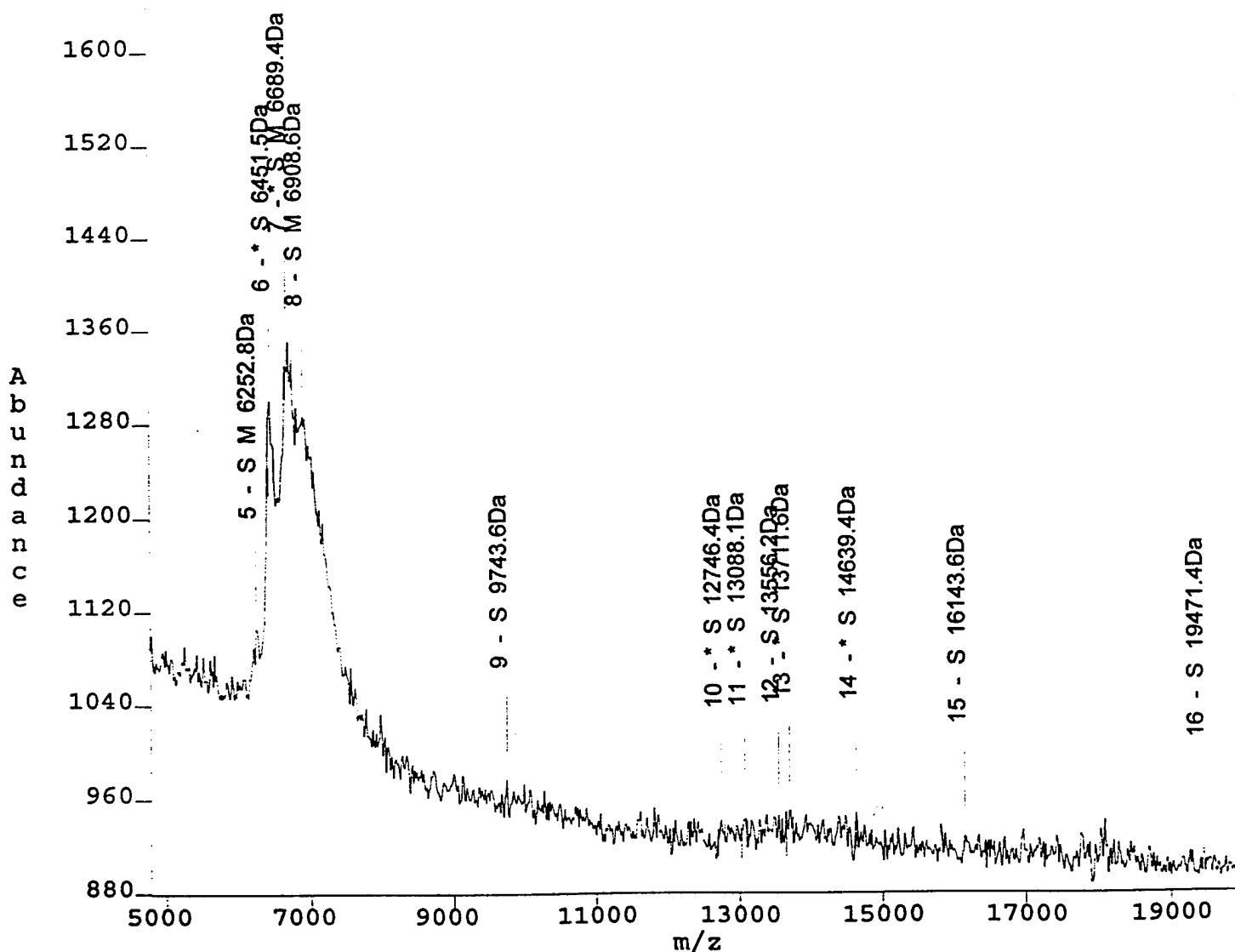
Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNNEG8.TOF [#2091]

## FIGURE 1D



NEW DATA\* [A.03.00]

#51321

Sample Name EGF/SANPAH 1:7.5  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/14/98

Collected Mon Apr 20 12:43:25 1998  
 Processed Mon Apr 20 12:52:40 1998  
 Printed Mon Apr 20 12:54:33 1998

Sequence C:\HPTOFLD\METHOD\PEP-NEG.MET\*

Method  
 Collection Mode Single Shots (55 of 263) Mesa 6 [14-117]  
 Laser Energy 3.06 (0.52) uJ Vacuum 7.03e-007 torr  
 Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

Peak	Height	Area	MW	delMW	%err	Name (page 1 of 1)
1 * S	3177	8684	1159.0			
2 * S	3442	14390	1182.1	23.1		
3 * S	2480	4930	1404.9	222.8		
4 * S	3230	12002	1428.1	23.2		
5 S M	1106	65	6252.8	4824.7		
6 * S	1300	5474	6451.5	198.7		
7 * S M	1351	2119	6689.4	238.0		
8 S M	1287	-64	6908.6	219.2		
9 S	977	553	9743.6	2835.0		
10 * S	941	665	12746.4	3002.8		
11 * S	943	702	13088.1	341.7		
12 S	945	543	13556.2	468.1		
13 * S	950	934	13711.6	155.4		
14 * S	947	509	14639.4	927.7		
15 S	927	545	16143.6	1504.2		
16 S	915	519	19471.4	3327.8		

\*=Gauss, (D)elected, (C)alibrant/(S)ample, M=Manual, P#=Polymer, ? = changed.

## FIGURE 1D (CONT'D)

Sign: \_\_\_\_\_

Date: / /

# FIGURE 1E

NEW DATA\* [A.03.00] #5119]  
 Sample Name EGF/SANPAH 1:~~7.5~~ 10  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application  
 4/14/98  
 Collected Sat Apr 18 14:52:38 1998  
 Processed Sat Apr 18 15:05:43 1998  
 Printed Sat Apr 18 15:06:10 1998  
 Sequence Method C:\HPTOFLD\METHOD\PEP-NEG.MET  
 Collection Mode Single Shots (57 of 106) Mesa 7 [41-98]  
 Laser Energy 2.95 (0.61) uJ Vacuum 4.10e-007 torr  
 Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None  
 A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010  
 Calibration - Program Calculated (2-Parameter)  
 Calibration Date Fri Nov 04 15:09:44 1994  
 Calibrator Christopher M. Adams  
 Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

2100—

1900—

1700—

1500—

1300—

1100—

2800 3500 4200 4900 5600 6300 7000 7700 8400 9100 9800  
m/z

NEW DATA\* [A.03.00] #5119]

Sample Name EGF/SANPAH 1:7.5 /D  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/14/98

Collected Sat Apr 18 14:52:38 1998  
 Processed Sat Apr 18 15:05:43 1998  
 Printed Sat Apr 18 15:06:10 1998

Sequence  
 Method C:\HPTOFLD\METHOD\PEP-NEG.MET

Collection Mode Single Shots (57 of 106) Mesa 7 [41-98]  
 Laser Energy 2.95 (0.61) uJ Vacuum 4.10e-007 torr  
 Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

Peak		Height	Area	MW	delMW	%err	Name (page 1 of 1)
1	*	S	3034	8516	1158.0		
2	*	S	2930	6516	1180.8	22.8	
3	*	S	3521	14052	1202.7	21.9	
4	*	S	3140	12575	1425.3	222.7	
5	*	S	3177	10810	1447.8	22.5	
6	*	S	1268	2433	6440.3	4992.4 - EGF/SAN (1:1)	
7	*	S	1458	2446	6682.2	242.0 - EGF/SAN (1:2)	
8	S	M	1375	1421	6880.2	198.0 - EGF/SAN (1:3)?	
9	S		1089	509	9908.4	3028.2	
10	S		1085	565	10795.9	887.4	
11	S		1062	937	12608.1	1812.2	
12	*	S	1059	658	13273.9	665.8	
13	S		1064	765	13384.4	110.5	
14	S		1064	772	14319.9	935.5	
15	*	S	1062	867	15132.0	812.1	
16	*	S	1047	517	15407.7	275.7	
17	*	S	1026	582	18507.7	3100.0	
18	S		1033	886	18622.7	115.0	
19	S		1026	544	19003.8	381.2	

\*=Gauss, (D)elected, (C)alibrant/(S)ample, M=Manual, P#=Polymer, ? = changed.

## FIGURE 1E (CONT'D)

Sign: \_\_\_\_\_

Date: / /

NEW DATA\* [A.03.00] #5108]

Sample Name EGF/Genistein 1:10      not pre-photolyzed  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/15/98

Collected Fri Apr 17 13:33:31 1998  
 Processed Fri Apr 17 13:35:31 1998  
 Printed Fri Apr 17 13:35:41 1998

Sequence Method C:\HPTOFLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 31.1) (50 of 113) Mesa 10 [57-59]  
 Laser Energy 1.76 (0.53) uJ Vacuum 6.17e-007 torr  
 Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

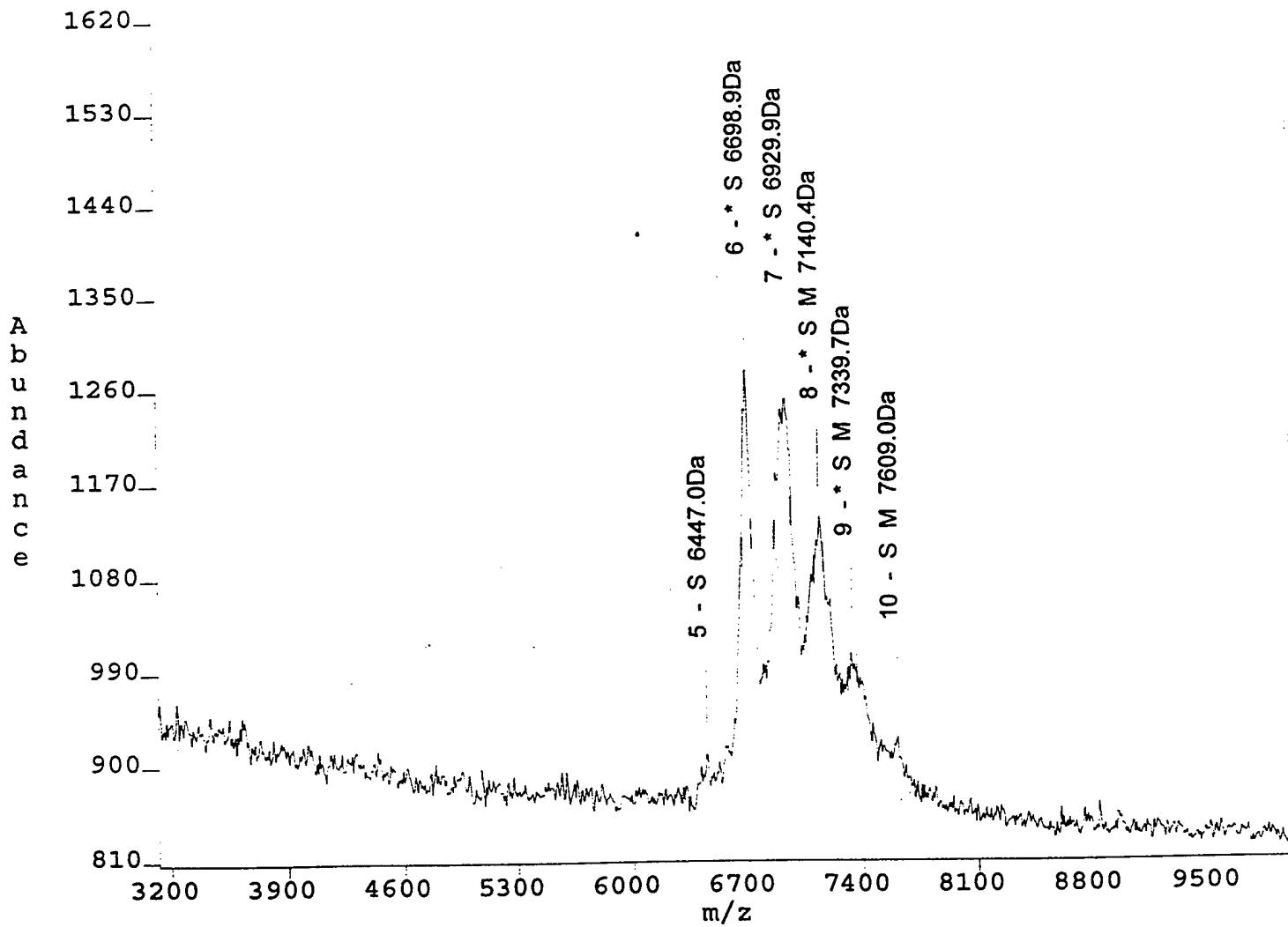
A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]



Sign: \_\_\_\_\_

Date: / /

**FIGURE 1F**

NEW DATA\* [A.03.00

#5108]

Sample Name EGF/Genistein 1:10  
 Preparation PBS  
 Matrix Sinnapinic Acid  
 User Name L. Ronken  
 Department Name Biotherapy  
 Application

4/15/98

Collected Fri Apr 17 13:33:31 1998  
 Processed Fri Apr 17 13:35:31 1998  
 Printed Fri Apr 17 13:35:41 1998

Sequence Method C:\HPTOFOLD\METHOD\PEP-NEG.MET

Collection Mode Auto Multi Shots (S/N 31.1) (50 of 113) Mesa 10 [57-59]  
 Laser Energy 1.76 (0.53) uJ Vacuum 6.17e-007 torr

Mass Range 20000 Da Ion Optics 28.0/7.0 kV  
 Mass Filter 350 Da Detector -4.75 kV  
 Data Interval 5.0 nsec Digitizer 1000 mVFS  
 Polarity Negative Filter None

A2 5.1885630 A1 -0.4177490 A0 0.0084090 res 16.1913010

Calibration - Program Calculated (2-Parameter)

Calibration Date Fri Nov 04 15:09:44 1994

Calibrator Christopher M. Adams

Calib Data File C:\HPTOF\DATA\PEPNEG8.TOF [#2091]

Peak		Height	Area	MW	delMW	%err	Name (page 1 of 1)
1	*	S	1491	3756	1075.8		
2	*	S	1771	7956	1119.5	43.7	
3	*	S	1387	6008	1208.1	88.6	
4	*	S	1283	1816	1454.3	246.2	
5	S		910	992	6447.0	4992.7 - EGF/SAN(1:1)	
6	*	S	1278	13601	6698.9	251.9 - EGF/SAN/Gen or EGF/SAN (1:2)	
7	*	S	1250	12509	6929.9	231.0 -	
8	*	S M	1137	2790	7140.4	210.5 -	
9	*	S M	1006	569	7339.7	199.3 -	
10	S	M	925	143	7609.0	269.3 -	

\*=Gauss, (D)elected, (C)alibrant/(S)ample, M=Manual, P#=Polymer, ? = changed.

## FIGURE 1F (CONT'D)

Sign: \_\_\_\_\_

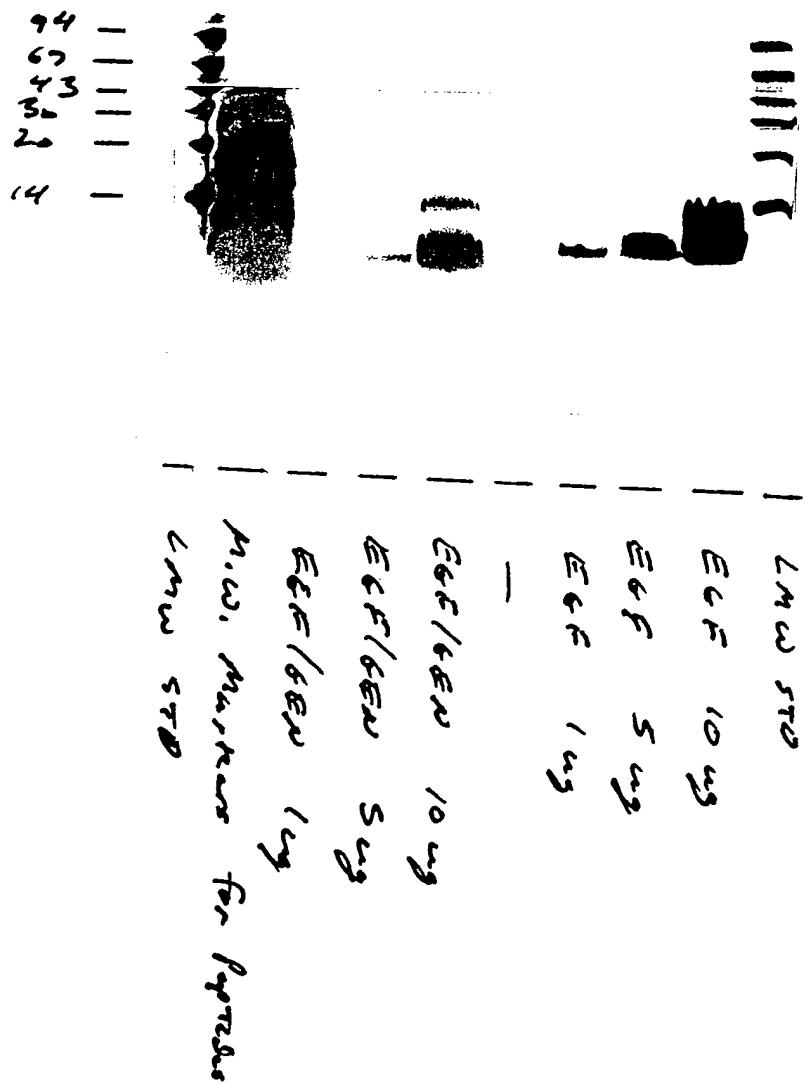
Date: / /

Tricaine salts

5-13-92

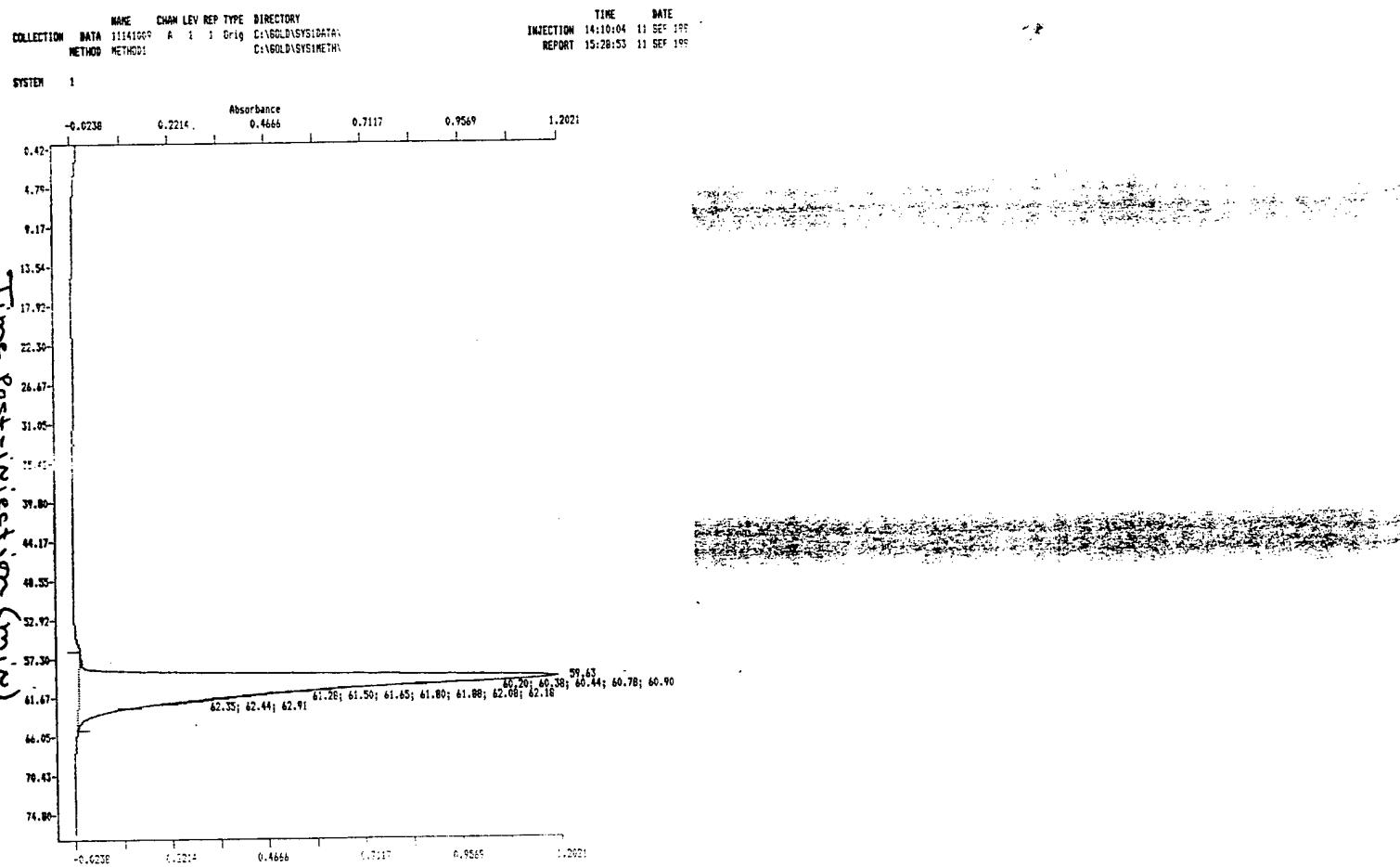
EGF & EGF/GEN

FIGURE 2



EGF

## FIGURE 3A

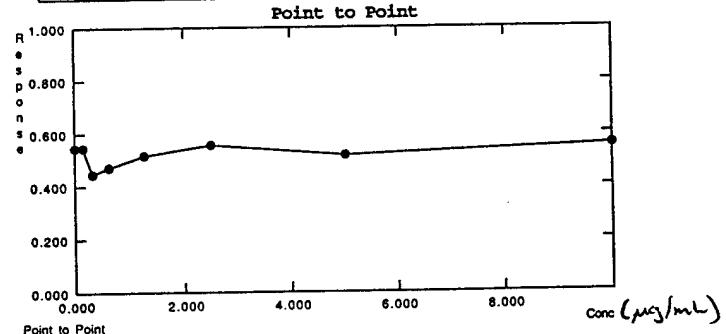


*EGF  
control for MTT*

## FIGURE 3A (CONT'D)

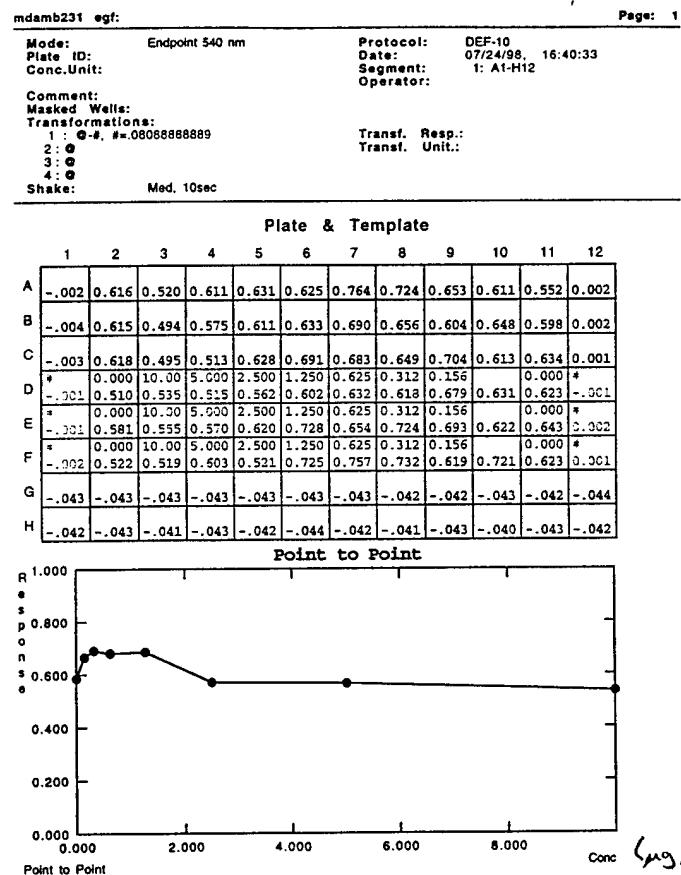
b120 EGF:												Page: 1
Mode: Endpoint 540 nm										Protocol: DEF-10		
Plate ID: 07/24/98, 16:07:14										Date: 07/24/98, 16:07:14		
Conc.Unit: 1: A1-H12										Segment: 1: A1-H12		
Comment:										Operator:		
Masked Wells:												
Transformations:										Transf. Resp.:		
1 : @-#, #=.1153333333										Transf. Unit.:		
2: @												
3: @												
4: @												
Shake: Med, 10sec												

Plate & Template												
1	2	3	4	5	6	7	8	9	10	11	12	
A 0.013	0.543	0.223	0.543	0.540	0.525	0.518	0.545	0.535	0.444	0.484	0.008	
B -.002	0.538	0.391	0.507	0.487	0.448	0.478	0.425	0.450	0.444	0.477	-.007	
C -.011	0.562	0.456	0.578	0.487	0.496	0.498	0.485	0.453	0.451	0.438	-.005	
D +.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	0.000	0.000	0.000	*
D -.011	0.565	0.523	0.496	0.523	0.523	0.438	0.454	0.628	0.481	0.463	-.012	
E +.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	0.000	0.000	0.000	*
E -.007	0.629	0.613	0.566	0.631	0.527	0.537	0.431	0.492	0.486	0.463	0.304	
F +.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	0.000	0.000	0.000	*
F 0.003	0.540	0.527	0.489	0.508	0.496	0.438	0.450	0.511	0.465	0.497	0.010	
G -.077	-.077	-.077	-.077	-.077	-.077	-.077	-.077	-.077	-.076	-.077	-.076	
H -.077	-.077	-.077	-.077	-.077	-.078	-.077	-.077	-.078	-.075	-.077	-.073	



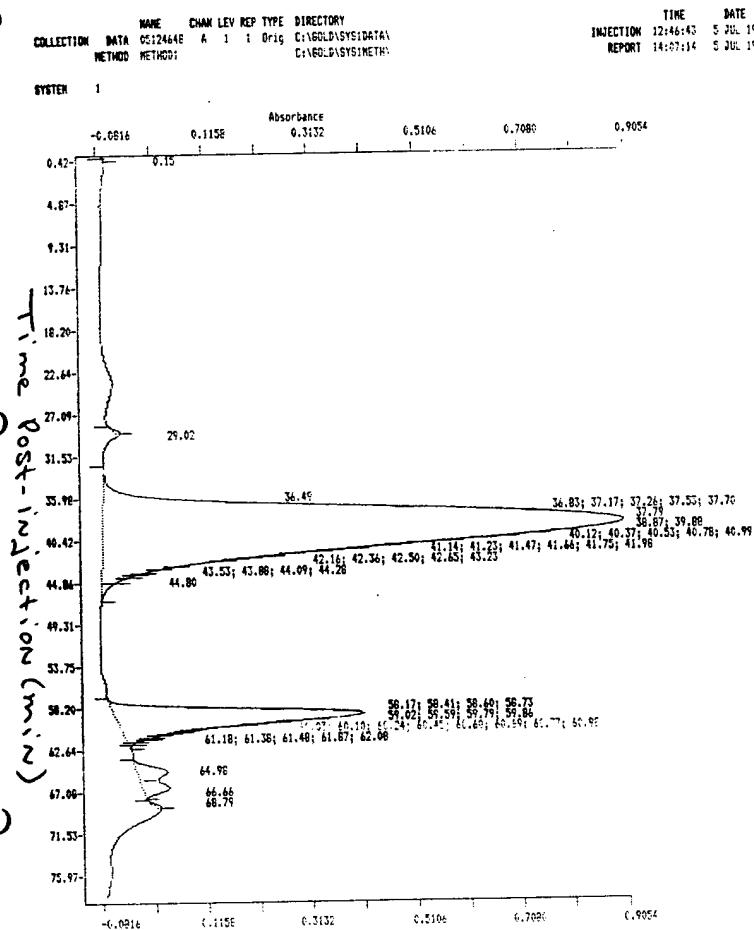
*EGF control  
for MT*

## FIGURE 3A (CONT'D)



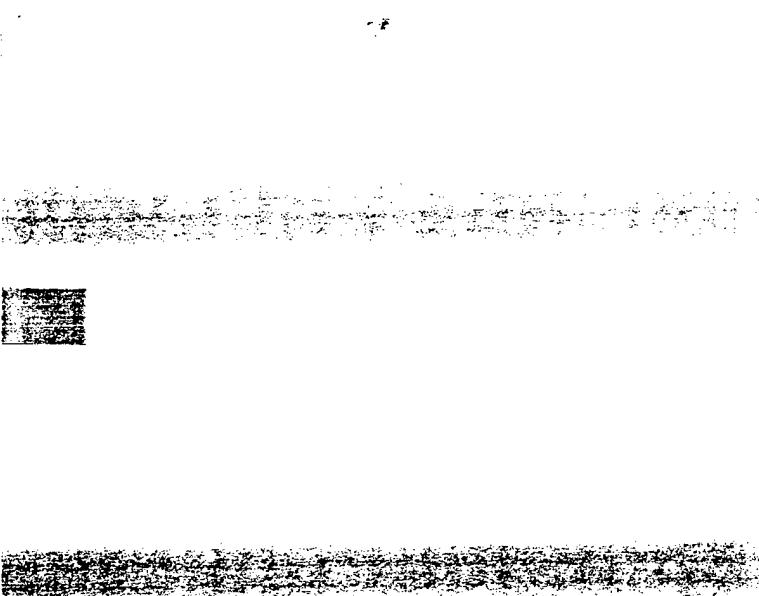
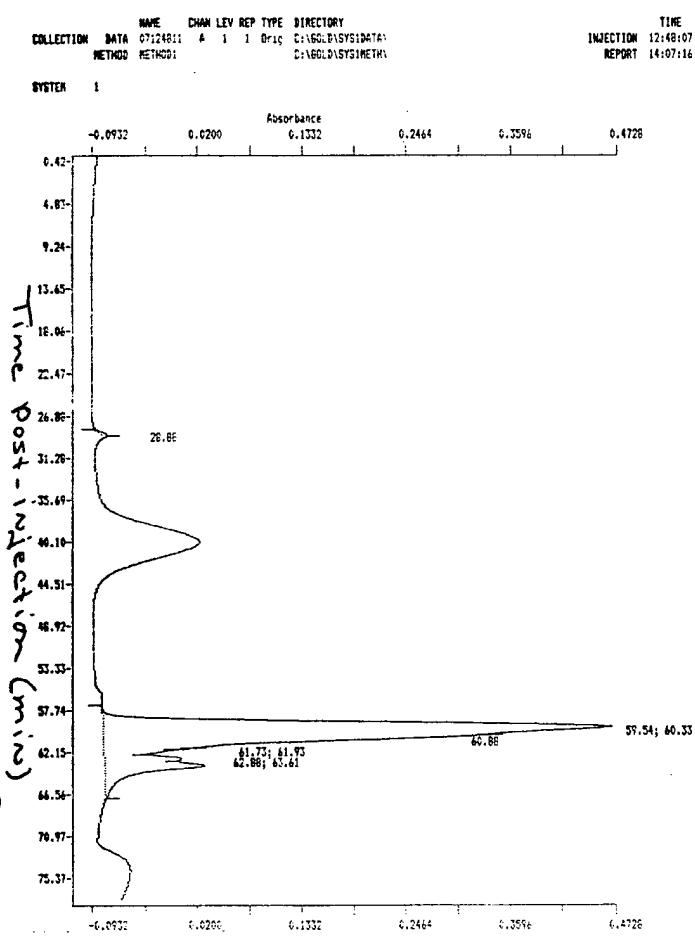
EGF/SAN-Gen  
(1:4)(1:20) pp

## FIGURE 3B



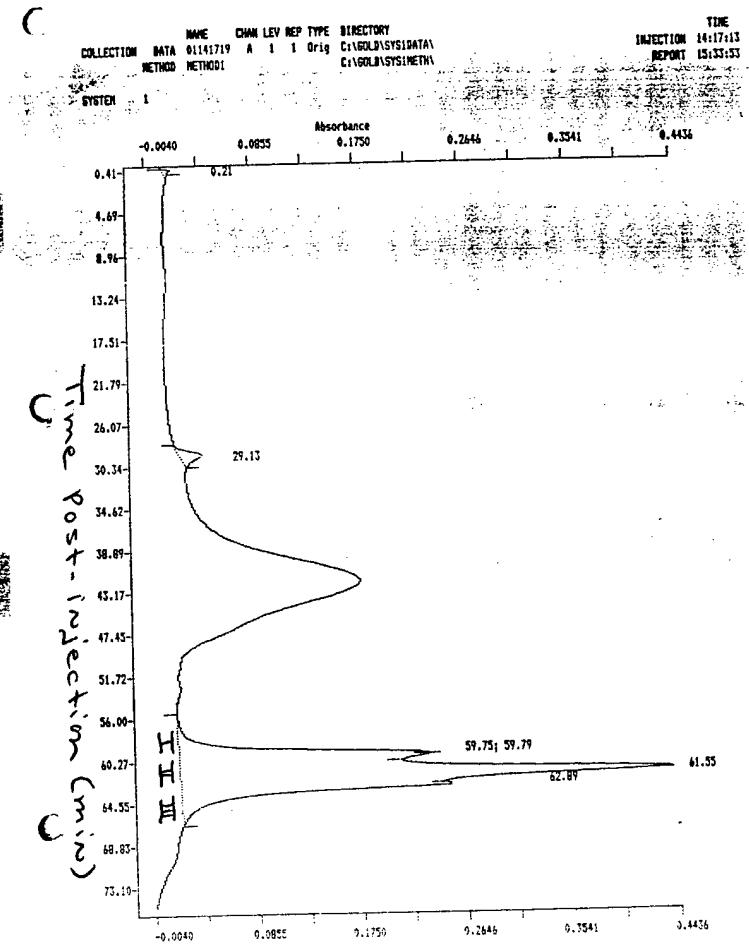
EGF/SAN-Gen  
(1:2)(1:20) PP

FIGURE 3C

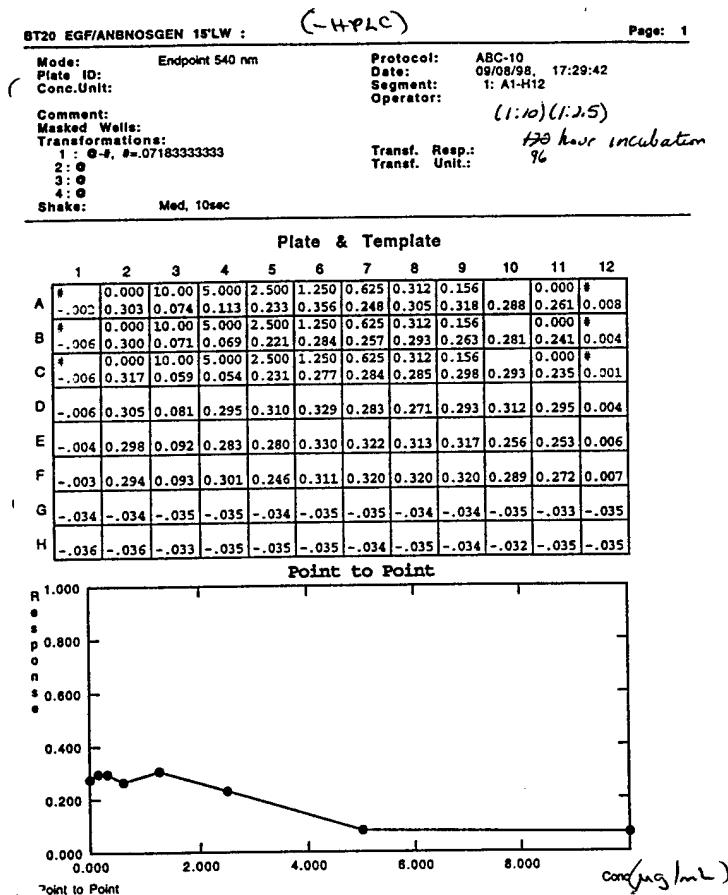


EGF/ANB-NOS-Gen  
(1:10)(1:2.5) PP

**FIGURE 4A**



# FIGURE 4A (CONT'D)



# FIGURE 4A (CONT'D)

Page: 1

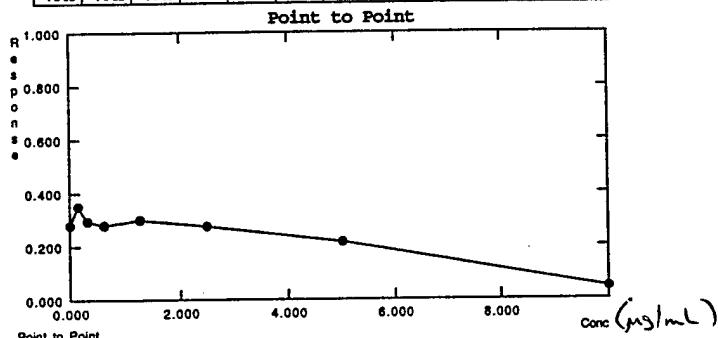
---

**BT20 EGF/ANBNOSEG 15'LWHPLCII:**

Mode:	Endpoint 540 nm	Protocol:	DEF-10
Plate ID:		Date:	09/08/98, 17:32:08
: Conc.Unit:		Segment:	1: A1-H12
Comment:		Operator:	(1:10) (1:25)
Masked Wells:			+20 hour incubation
Transformations:		Transf. Resp.:	96
1: @-, #=.051		Transf. Unit.:	
2: @			
3: @			
4: @			
Shake:	Med, 10sec		

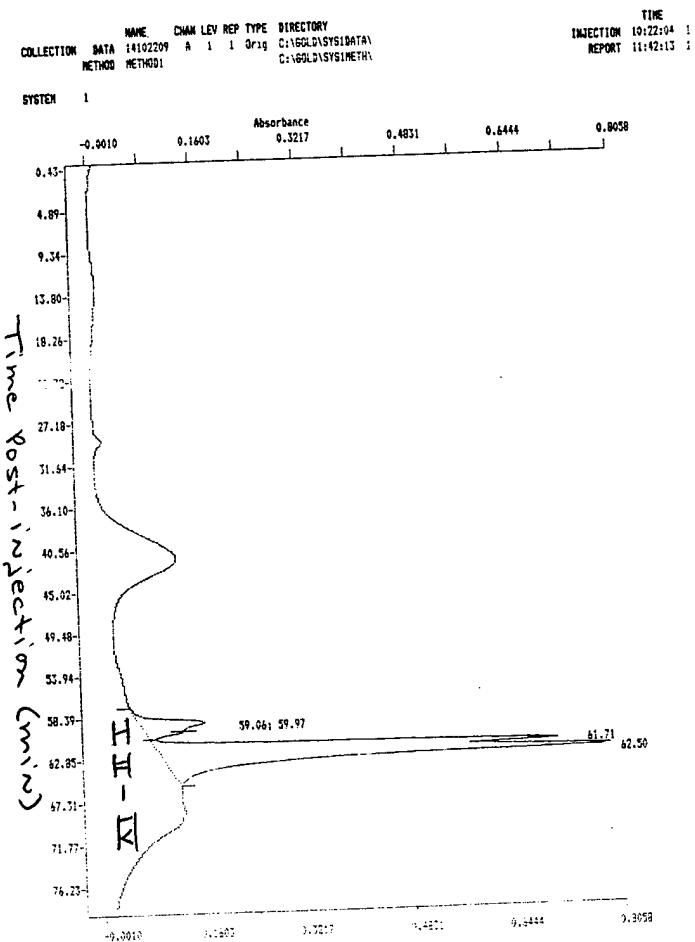
---

Plate & Template												
1	2	3	4	5	6	7	8	9	10	11	12	
A -.009	0.278	0.281	0.273	0.276	0.383	0.229	0.245	0.279	0.225	0.218	-.003	
B -.011	0.336	0.244	0.309	0.297	0.299	0.285	0.289	0.316	0.220	0.253	-.009	
C -.012	0.250	0.238	0.281	0.305	0.261	0.290	0.263	0.349	0.282	0.285	-.007	
D * 0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	*			
D -.015	0.281	0.040	0.196	0.234	0.256	0.268	0.294	0.370	0.250	0.237	-.008	
E * 0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	*			
E -.013	0.301	0.047	0.210	0.290	0.299	0.298	0.313	0.362	0.293	0.255	-.304	
F * 0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	*			
F -.006	0.313	0.051	0.245	0.296	0.338	0.279	0.278	0.324	0.309	0.287	0.046	
G -.044	-.045	-.043	-.042	-.041	-.043	-.042	-.042	-.042	-.042	-.042	-.044	-.044
H -.043	-.043	-.043	-.043	-.043	-.043	-.043	-.042	-.042	-.040	-.044	-.044	-.044



EGF/ANB-NOS-Gen  
(1:10)(1:10) PP

## FIGURE 4B



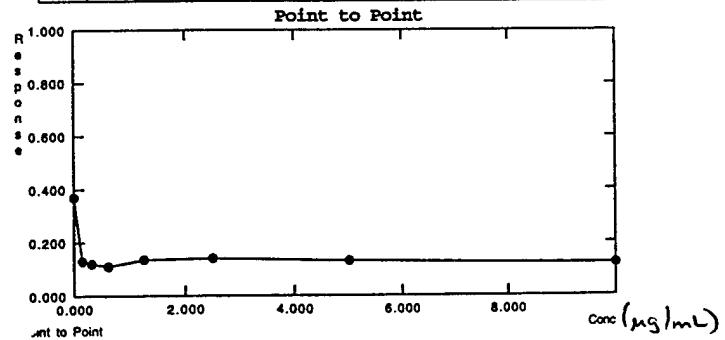
## FIGURE 4B (CONT'D)

BT20-EQFANBNOSEnHPLCII7/14/98:

Page: 1

Code:	Endpoint 540 nm	Protocol:	ABC-10
late ID:		Date:	07/17/98, 17:17:02
Conc.Unit:		Segment:	1: A1-H12
Comment:		Operator:	48 hours
Masked Wells:			
Transformations:			
1 : @	#=.1118333333	Transf. Resp.:	
2 : @		Transf. Unit.:	
3 : @			
4 : @			
Shake:	Med, 10sec		

Plate & Template												
	1	2	3	4	5	6	7	8	9	10	11	12
A	#	0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	*	
	-.009	0.333	0.120	0.109	0.123	0.136	0.115	0.125	0.133	0.351	0.389	.003
B	#	0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	*	
	0.009	0.368	0.124	0.145	0.157	0.135	0.108	0.119	0.119	0.358	0.372	.001
C	#	0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	*	
	-.001	0.378	0.114	0.143	0.142	0.133	0.104	0.116	0.140	0.429	0.383	-.004
D	-.024	0.368	0.126	0.135	0.145	0.143	0.113	0.114	0.150	0.363	0.401	-.001
E	0.137	0.358	0.148	0.145	0.152	0.152	0.124	0.120	0.148	0.355	0.411	0.002
	-.002	0.362	0.150	0.135	0.148	0.144	0.121	0.048	0.139	0.347	0.340	0.004
G	-.074	-.073	-.074	-.074	-.074	-.074	-.073	-.074	-.073	-.074	-.074	-.074
H	-.075	-.075	-.074	-.074	-.075	-.074	-.074	-.075	-.074	-.073	-.073	-.075

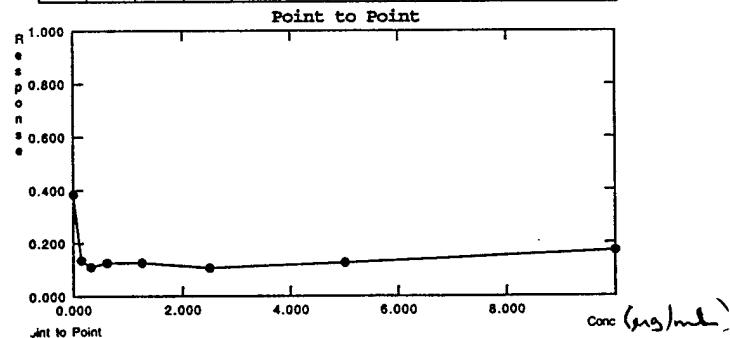


## FIGURE 4B (CONT'D)

MDAMB231-EGFANBNOSGenHPLCIII15: 7/17/98 Page: 1

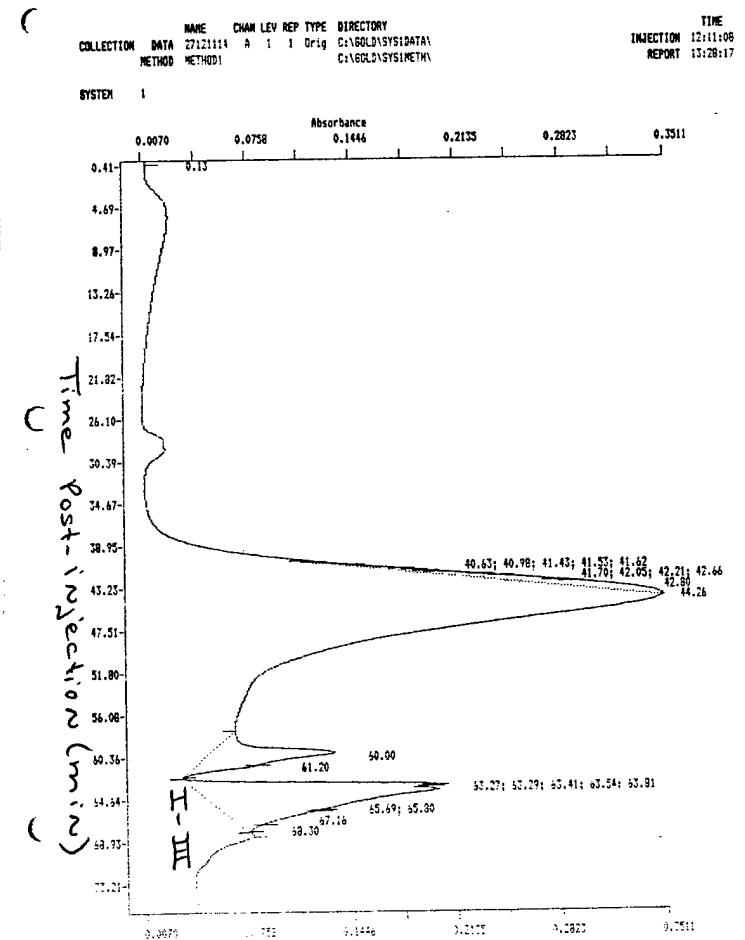
Mode: Endpoint 540 nm Protocol: ABC-10  
 Date ID: 07/17/98, 17:36:40  
 Conc. Unit: Segment: 1: A1-H12  
 Operator: 48 hours  
 Comment:  
 Masked Wells:  
 Transformations:  
 1: 0-.#,.00685 Transf. Resp.:  
 2: 0 Transf. Unit.:  
 3: 0  
 4: 0  
 Shake: Med, 10sec

Plate & Template													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	4 0.000	0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	#	0.303	
B	4 -.004	0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	#	-.004	
C	4 0.013	0.000	10.00	5.000	2.500	1.250	0.625	0.312	0.156	0.000	#	-.008	
D	4 0.002	0.417	0.307	0.207	0.230	0.122	0.131	0.128	0.252	0.401	0.410	-.000	
E	4 -.006	0.000	0.358	0.314	0.229	0.213	0.125	0.186	0.113	0.191	0.369	0.417	-.002
F	4 -.030	0.000	0.396	0.333	0.207	0.222	0.140	0.206	0.131	0.204	0.401	0.405	-.008
G	4 -.031	0.000	-.029	-.030	-.029	-.030	-.029	-.028	-.029	-.030	-.027	-.030	-.031
H	4 -.031	0.000	-.031	-.030	-.029	-.030	-.031	-.030	-.030	-.030	-.027	-.030	-.030

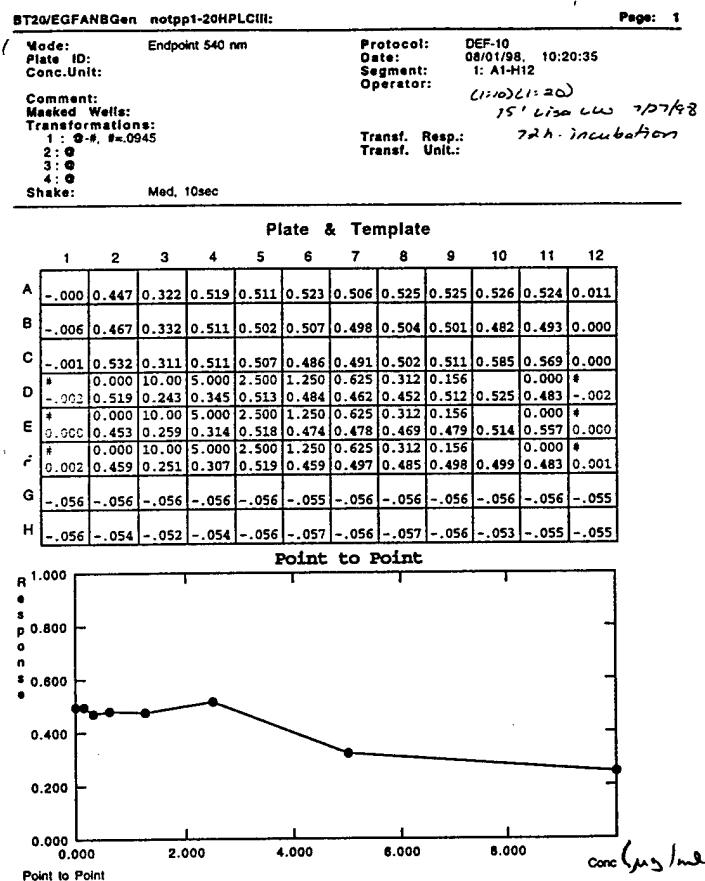


**FIGURE 4C.**

EGF/ANB-NOS-Gen  
(1:10)(1:20) Not PP



## FIGURE 4C (CONT'D)

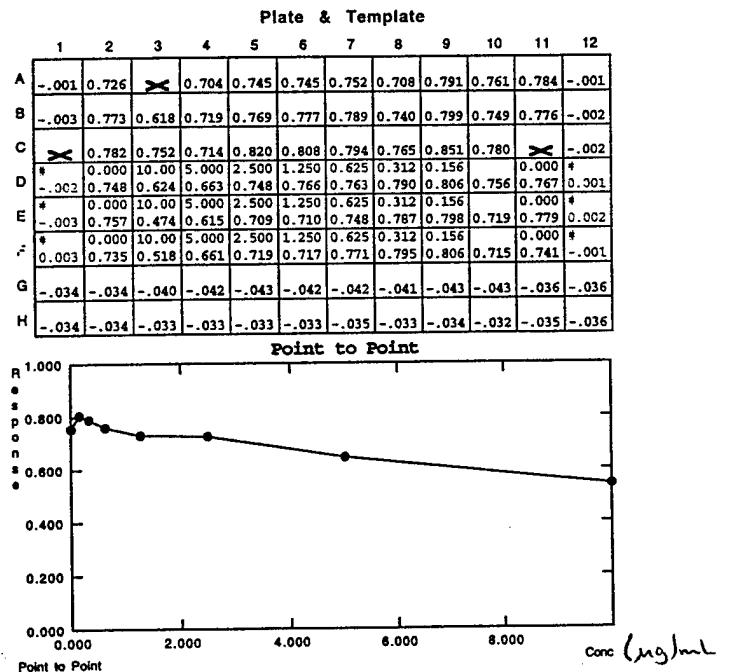


## FIGURE 4C (CONT'D)

Page: 1

**MDAMB231EGFANBGen nopp HPLCIII:**

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Plate ID:		Date:	08/01/98, 10:42:17
Conc.Unit:		Segment:	1: A1-H12
Comment:		Operator:	Lisa CW not PJS U:\o\21\203
Masked Wells:	A3 C1 C11		15' Lisa CW not PJS 7/27/98
Transformations:		Transf. Resp.:	72h. incubation
1: G-, #=.073		Transf. Unit:	
2: G			
3: G			
4: G			
Shake:	Med, 10sec		



# FIGURE 5

Date: 10/06/98

Gel #: EGF A2

PAGE 3.5%

Buffer System: Laemmli

Tricine

Other \_\_\_\_\_

Lane	Sample	Volume (ul)
1	Lmaw 500	1.0
2	Lmaw 500	1.0
3	EGF/m <sup>*</sup> (1:10) 1mL w 0.2ug	2.5
4	" " -HPLC 0.5ug	6.3
5	EGF/m 16cm HPLC II 0.2ug	3.4
6	" " " 0.5ug	8.5
7	EGF 1m/16cm HPLC II 0.2ug	10.0
8	" " " 0.5ug	25.0
9	EGF/m (1:10) 10/5 0.2ug	1.1
10	" " " 0.5ug	2.9
11		
12		
13		
14		
15		

STRATAGENE EAGLEYE II 10/07/98 05:07:30

EGF-GEN 10.06.98

IMAGE SIZE (640 x 480 x 8).

REAL-TIME ACQUIRE.

IMAGE CREATED ON WED OCT 07 05:06:32 1998.

Run: \_\_\_\_ volts 50mA 2-3 hours

Blot: NC PVDF

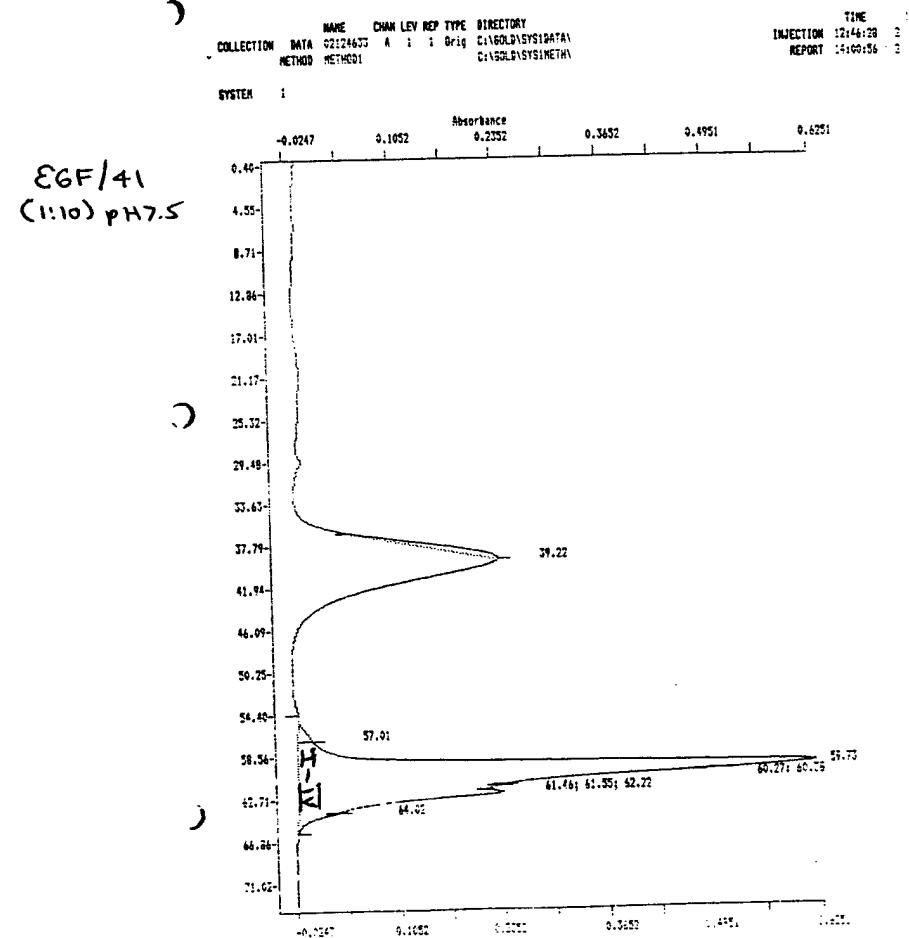
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1 Ab: \_\_\_\_\_  
Incubation: \_\_\_\_ @ \_\_\_\_ C

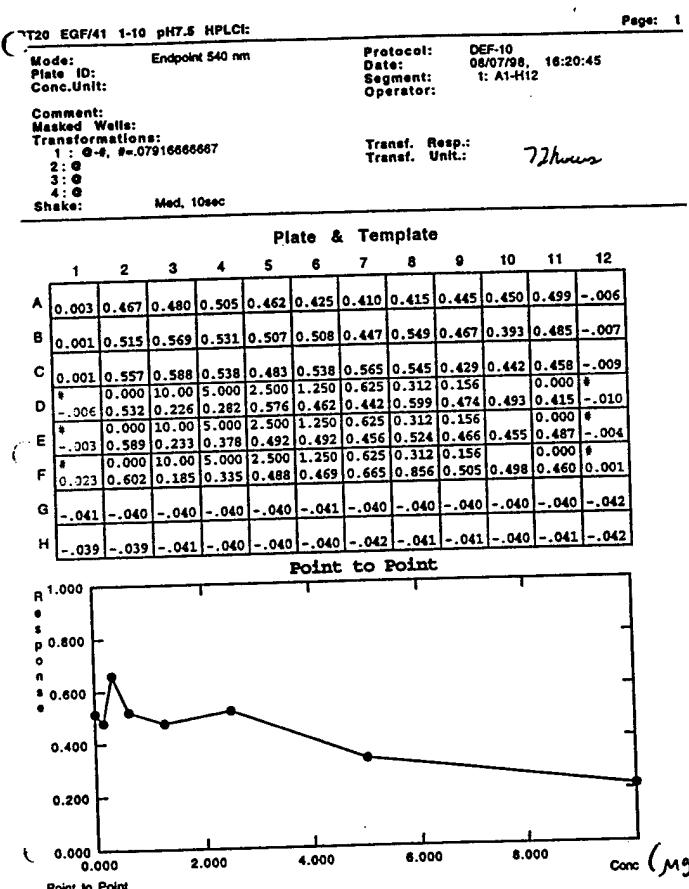
2 Ab: \_\_\_\_\_  
Incubation: \_\_\_\_ @ \_\_\_\_ C



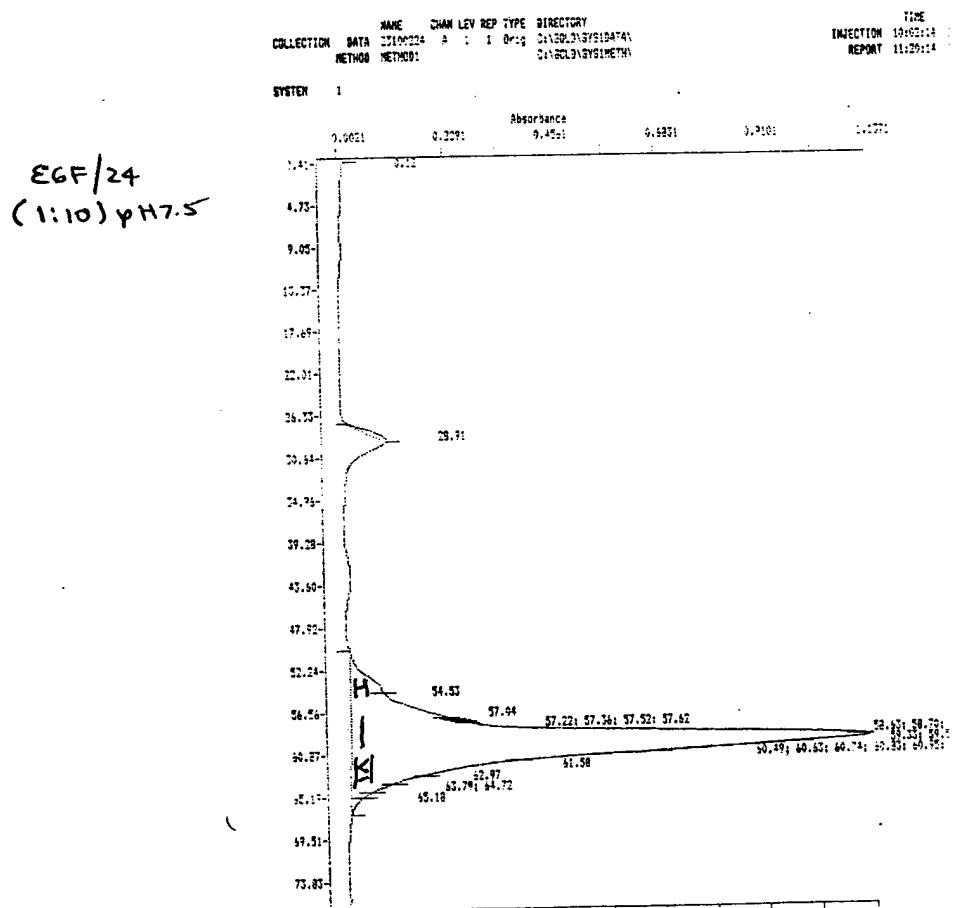
**FIGURE 6A**



## FIGURE 6A (CONT'D)

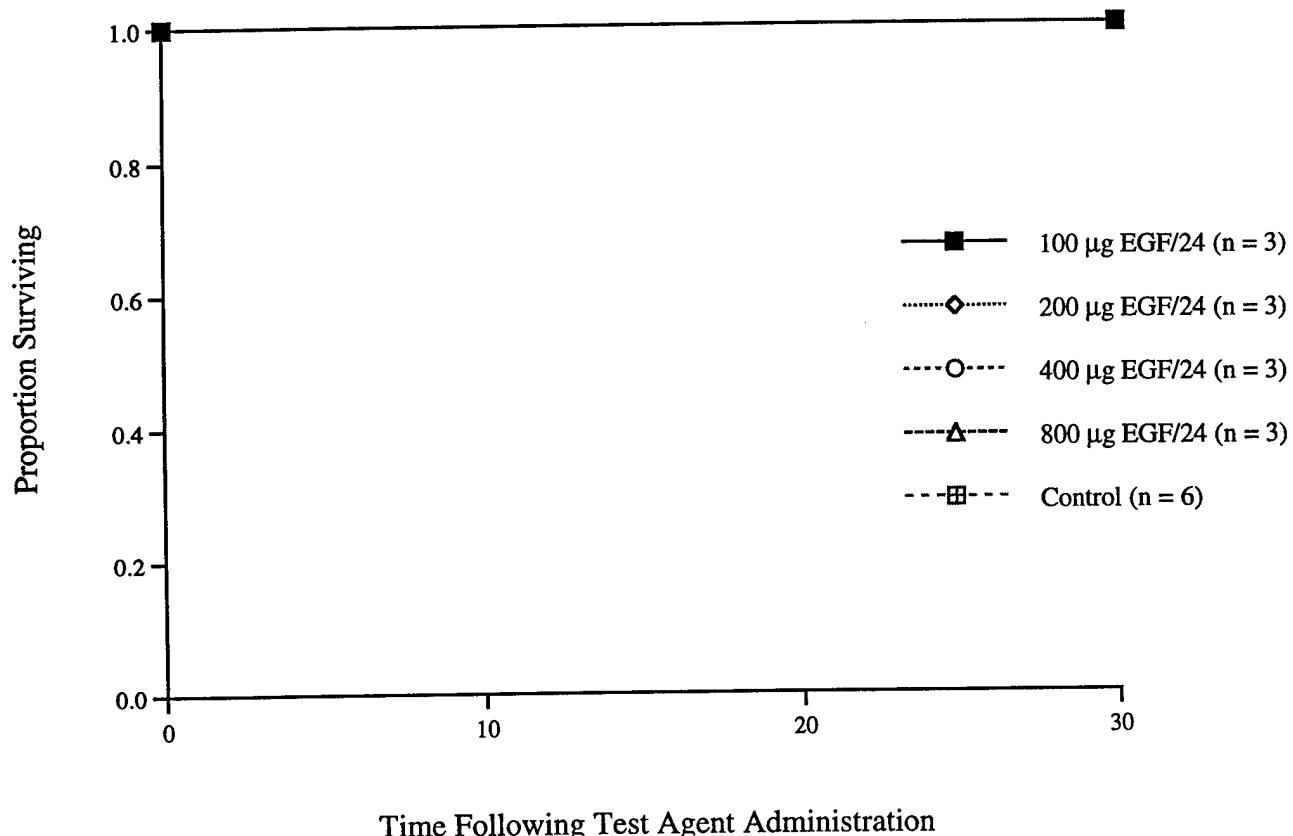


## FIGURE 6B

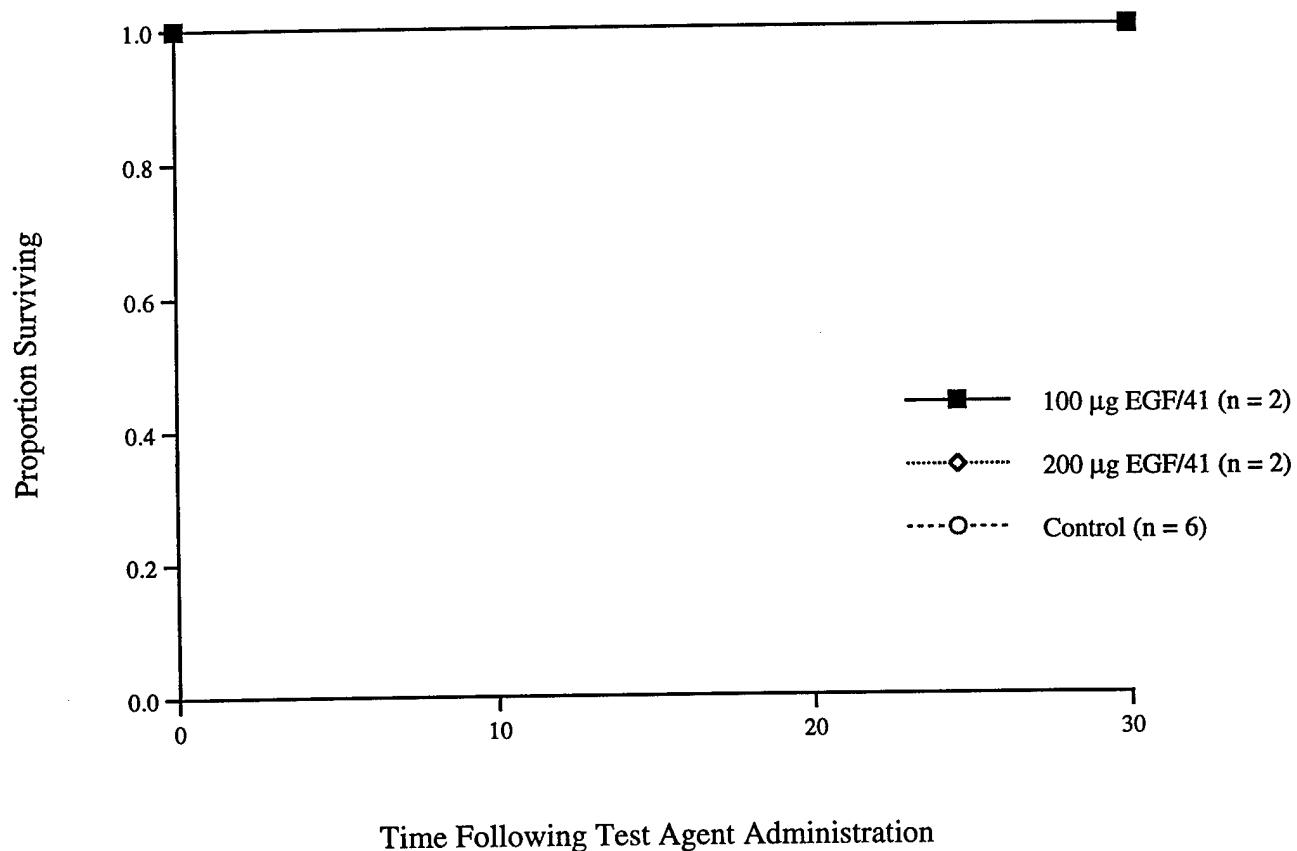


**FIGURE 7A**

**Effect of EGF/24 on Survival of Balb/c Mice**

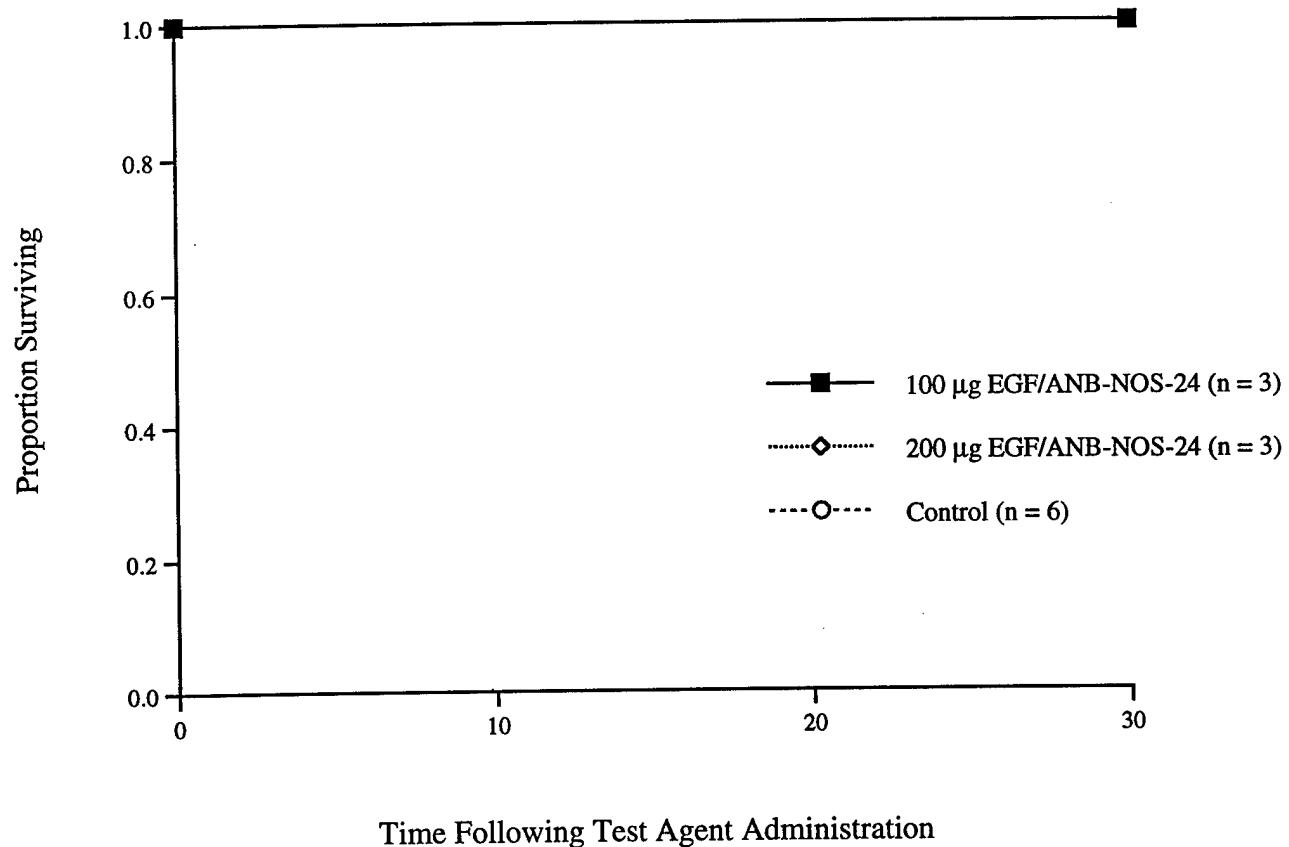


**FIGURE 7B**  
**Effect of EGF/41 on Survival of Balb/c Mice**

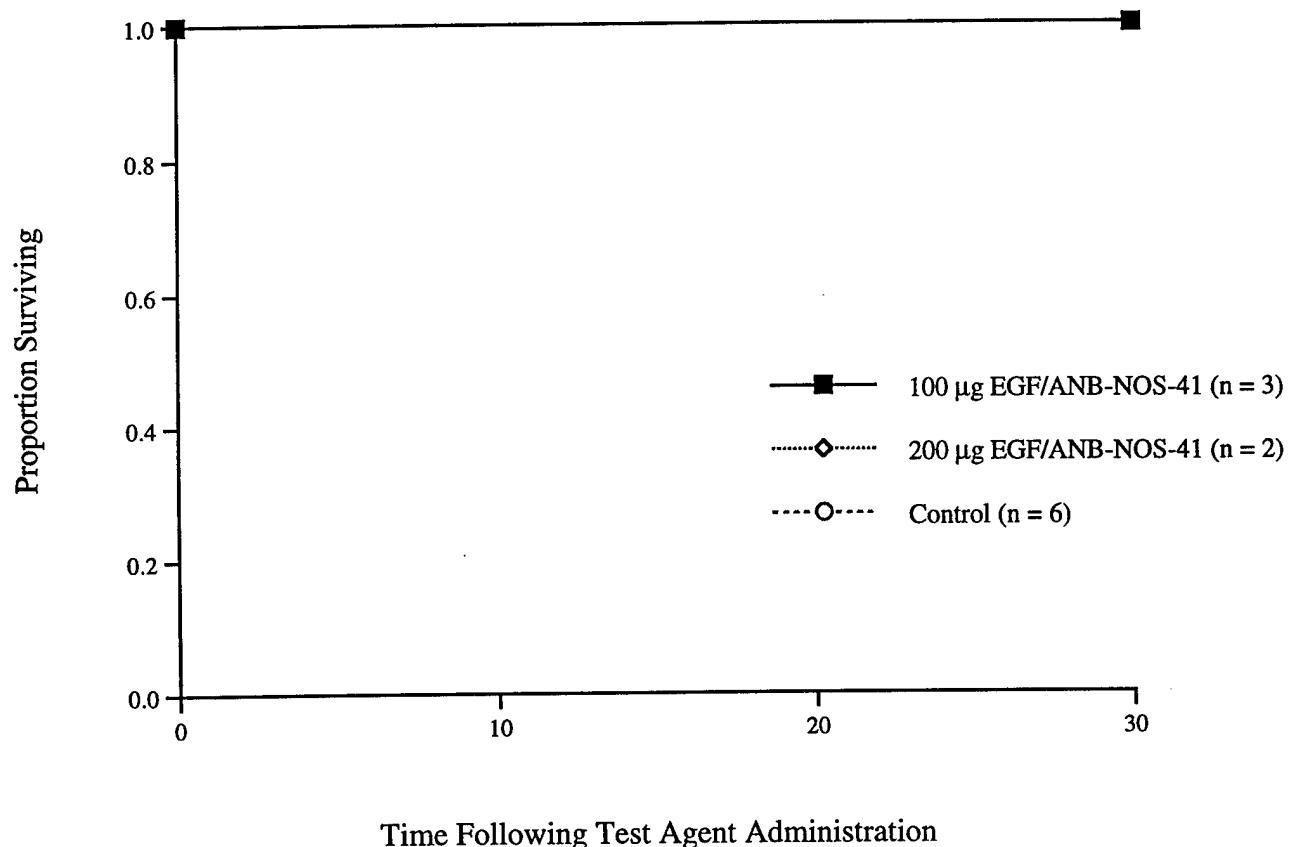


## **FIGURE 7C**

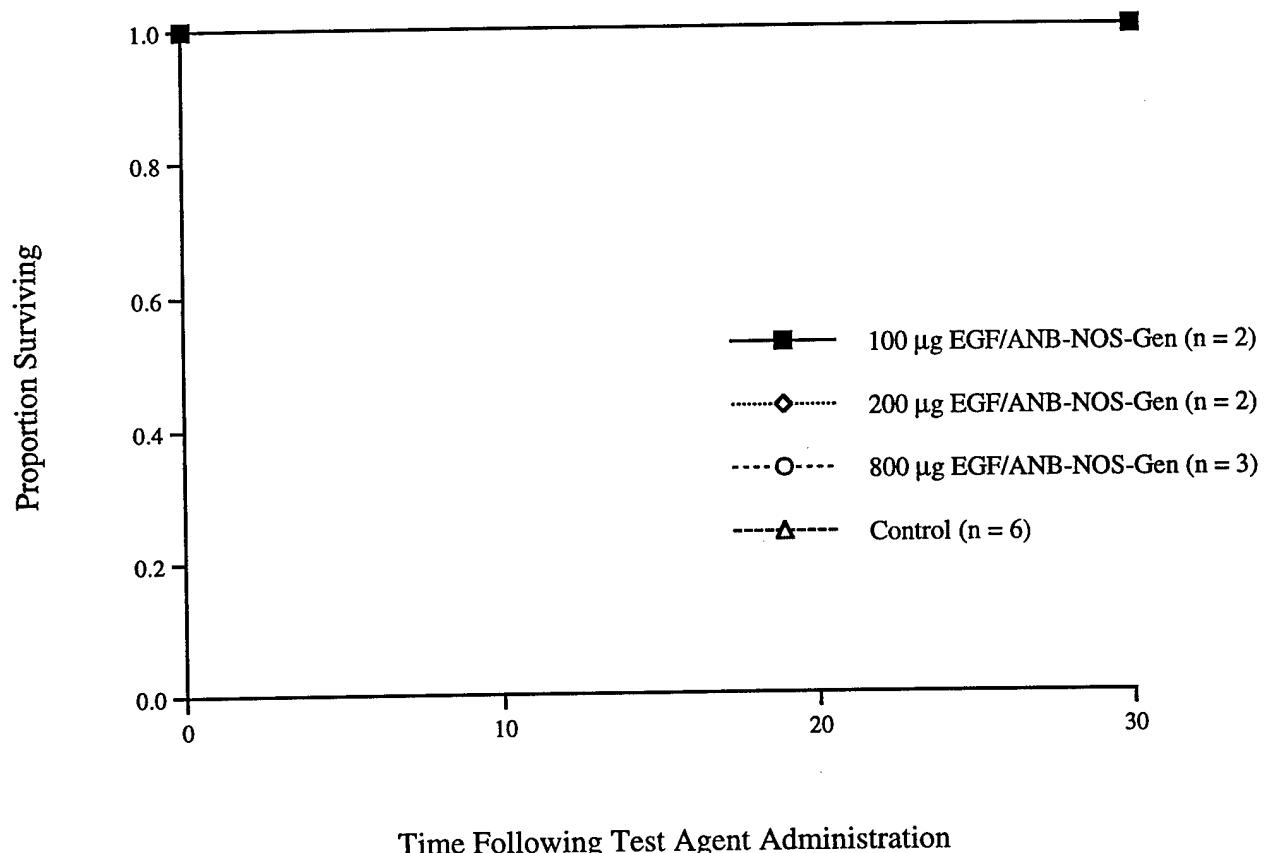
### **Effect of EGF/ANB-NOS-24 on Survival of Balb/c Mice**



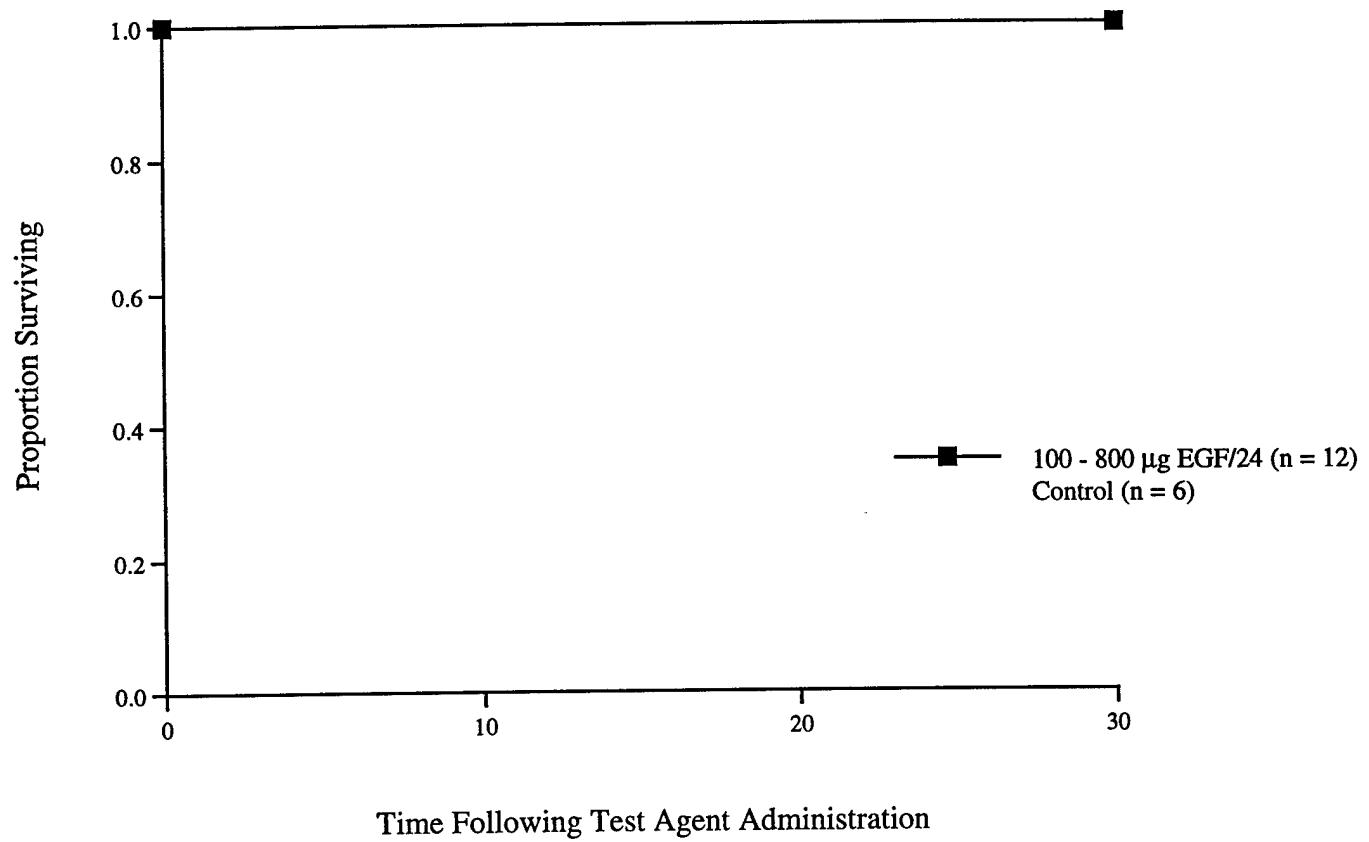
**FIGURE 7D**  
**Effect of EGF/ANB-NOS-41 on Survival of Balb/c Mice**



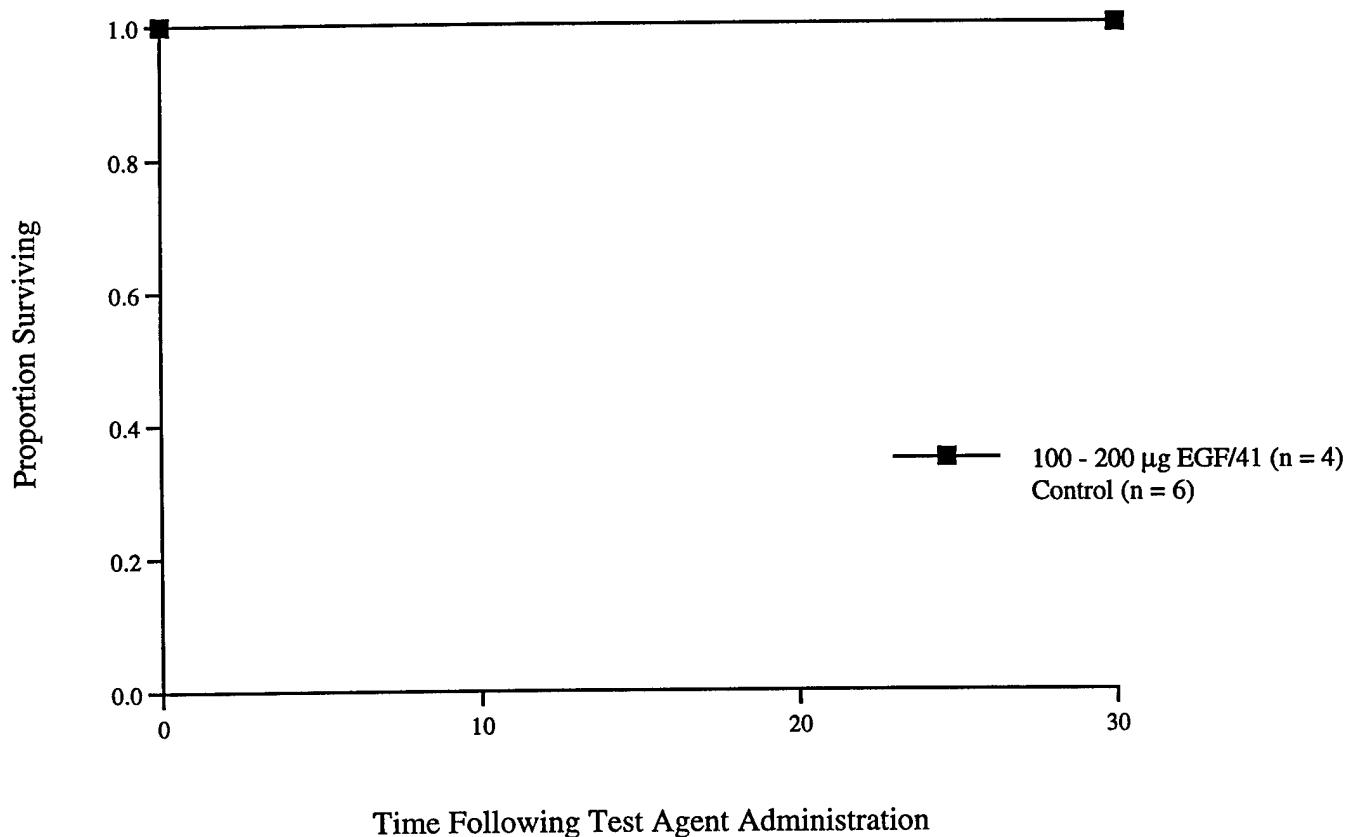
**FIGURE 7E**  
**Effect of EGF/ANB-NOS-Gen on Survival of Balb/c Mice**



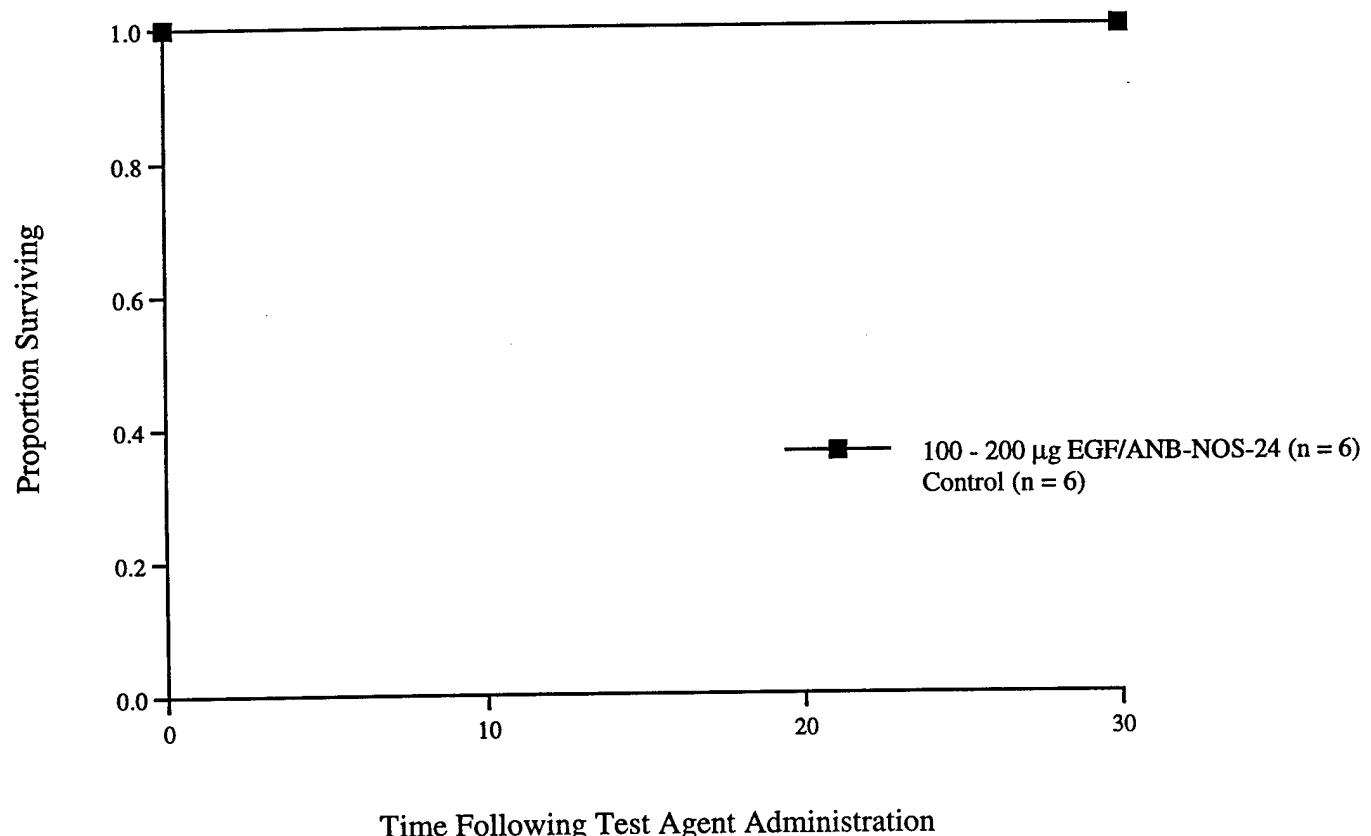
**FIGURE 7F**  
**Effect of EGF/24 on Survival of Balb/c Mice**



**FIGURE 7G**  
**Effect of EGF/41 on Survival of Balb/c Mice**

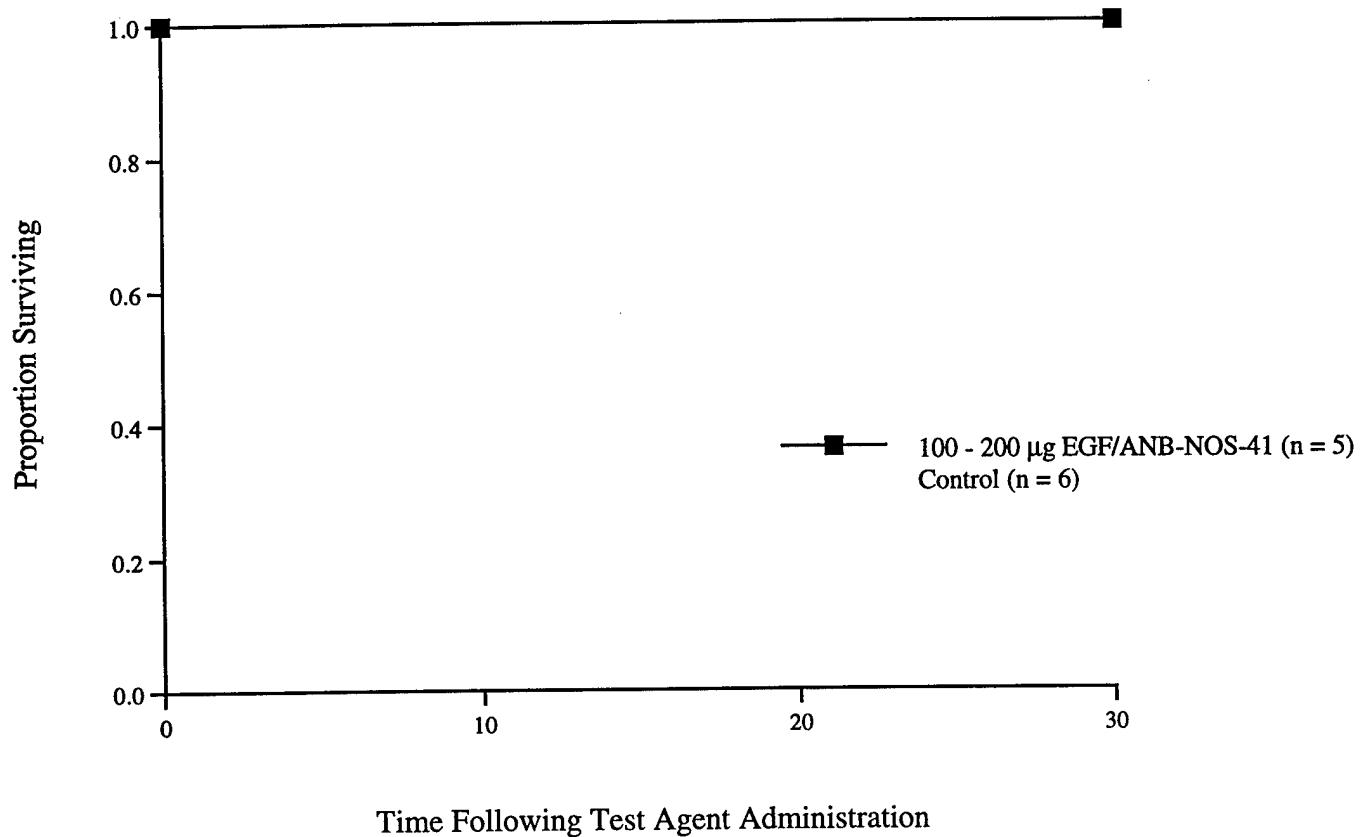


**FIGURE 7H**  
**Effect of EGF/ANB-NOS-24 on Survival of Balb/c Mice**

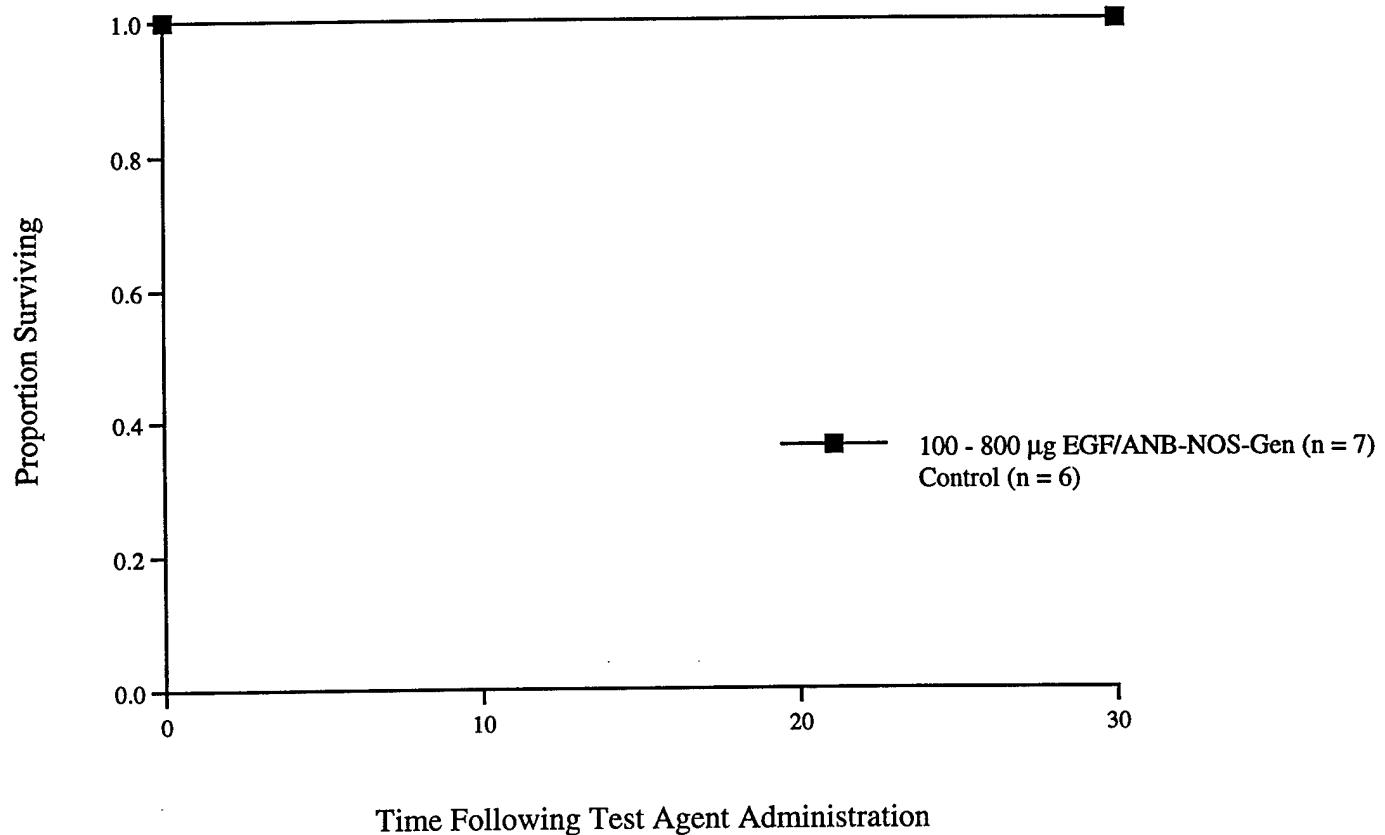


## **FIGURE 7I**

### **Effect of EGF/ANB-NOS-41 on Survival of Balb/c Mice**



**FIGURE 7J**  
**Effect of EGF/ANB-NOS-Gen on Survival of Balb/c Mice**



## **DOCUMENT 3. Year 1999 Annual Report**

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## **INTRODUCTION**

We have continued our efforts to optimize the design of the EGF-Genistein and related tyrosine kinase inhibitor conjugates. The goal of these continuing efforts is to prepare a new generation of EGF conjugates with unprecedented activity as well as stability. The design optimization represents work done at the Hughes Institute whereas the mouse and monkey studies are being conducted at the University of Minnesota. The work as well as analyses are ongoing and no conclusions are yet possible as to whether or not the novel EGF conjugates will be superior to the first generation EGF conjugates. Depending on these results, we will pick the most promising conjugate and start its use as part of combined biochemotherapy regimens, as originally proposed in our application.

## **BODY**

### **SECTION I: DESIGN OPTIMIZATION**

### **MATERIALS AND METHODS**

**Preparation of EGF-Genistein and Related Conjugates .** rhEGF was produced in *E. coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies and the mature protein was recovered by trypsin-cleavage followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of  $4.6 \pm 0.2$ . The endotoxin level was 0.17 EU/mg.

The recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) was initially employed in the synthesis of the EGF-Genistein(Gen) conjugates. Sulfo-SANPAH was dissolved in DMSO and used to modify EGF at molar ratios of 1:1 - 1:10, EGF to crosslinker. Following size-exclusion chromatography to remove unreacted crosslinker and small molecular weight reaction products, the modified rhEGF was mixed with a 10:1 or 20:1 molar ratio of Gen (LC Laboratories, Woburn, MA) [50 mM solution in dimethyl sulfoxide (DMSO)] and then irradiated for 10 - 60 min with long-wave UV light ( 366 nm Model UVGL-58 Mineralight; UVP, Upland, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a molar excess of Genistein resulted in the attachment of Gen to lysine 28, lysine 48, or the N-terminal residue of EGF. Excess Gen in the reaction mixture was removed by passage through a G25-Sephadex prepacked column.

The EGF- Gen conjugate was subsequently filter-sterilized and the protein concentration determined using the Bicinchoninic Acid(BCA) Protein Assay kit obtained from Sigma Chemical Company. Bicinchoninic acid is a chromogenic reagent, highly specific for Cu(I), which forms a purple complex with an absorbance at 562 nm that is directly proportional to the protein concentration.

In addition to Sulfo-SANPAH, we also used the following crosslinking agents obtained from Pierce Chemical Company: N-5-azido-2-nitrobenzoyloxysuccinimide(ANB-NOS), Sulfosuccinimidyl 2-[m-azido-o-nitrobenzamido]ethyl-1,3'-dithiopropionate(SAND), and Sulfosuccinimidyl(perfluoroazidobenzamido)ethyl-1,3-dithiopropionate(SFAD). These crosslinkers are of different chain lengths, ANB-NOS being the shortest at 7.7 Å, and all have a phenyl azide at one end

to react with Genistein following photolysis. The other end of the crosslinker contains an N-hydroxysuccinimide ester to react with protein amino groups. SAND and SFAD are cleavable by thiols.

To avoid exposing EGF to the possible harmful effects of UV light, we have also photolyzed the crosslinker-Genistein mixture prior to the addition of EGF. We dissolved both the crosslinker and Genistein in DMSO and mixed them together using a 20:1, 10:1, 5:1, or 2.5:1 molar ratio of Genistein to crosslinker. Photolysis was performed at room temperature for periods of time from 15 minutes to 48 hr using either a Model UVM-57(302 nm mid-range wavelength) or Model UVGL-58(366 nm longwave) UV lamp from UVP(Upland, CA). Following photolysis, the mixture was added to a solution of EGF(in PBS) at molar ratios of 2:1 to 10:1, crosslinker:EGF in a maximum final DMSO concentration of 10%.

In an effort to generate more potent EGF conjugates, we have also attempted to link other compounds which have themselves been shown to possess cytotoxic activity in in vitro systems. These compounds include two Genistein analogues, DDE24 and DDE41, which have been modified to contain an N-hydroxysuccinimide ester for direct conjugation to EGF in the absence of photolysis. We have also employed the above photolysis procedures to form EGF conjugates of the novel quinazoline derivatives, WHI-P97 and WHI-P154, as well as of DDE24 and DDE41.

**HPLC Analysis.** Reverse phase HPLC using a Hewlett-Packard (HP) 1100 series HPLC instrument was used to monitor and characterize the EGF-Gen conjugations. Analytical HPLC was performed using a LiChrospher 100(RP-18, 5 um) reverse phase column (250x4 mm, Hewlett-Packard). HPLC chromatograms were run at wavelengths of 220 nm, 280 nm, 308 nm, or 480 nm using the multiple wavelength detector option supplied with the instrument. UV spectra were generated for the individual peaks of

interest in the chromatogram. Five - 100 uL samples were applied to the above column and analysis was achieved using a gradient flow as follows: t = 0, 20% D; t = 5, 30% D; t = 9, 38% D; t = 20, 43.5% D; t = 35, 100% D; t = 50, 100% D; t = 55, 20% D. Eluent A consisted of a mixture of 0.1% trifluoroacetic acid(TFA) in water and eluent D contained 80% acetonitrile (CH<sub>3</sub>CN), 20% H<sub>2</sub>O, and 0.1% TFA.

Size-exclusion chromatography was carried out using a Beckman System Gold Instrument equipped with either a preparative TSKG3000SW column equilibrated in 100 mM sodium phosphate buffer, pH 6.8 at a flow rate of 3 mL/minute or an analytical TSKG3000PW column run in the same buffer at a flow rate of 0.2 mL/min

**Mass Spectrometry.** Mass spectrometric analysis was routinely performed to determine the relative molecular weights of the modified EGF and EGF-Genistein conjugates using a Hewlett-Packard Model G2025A matrix-assisted laser desorption/ionization mass spectrometer with linear time-of-flight mode (MALDI-TOF). In conjunction with the Hewlett-Packard instrument were a sample preparation assembly model G2024A including a high vacuum pump and a Dos-Chem station controller model G1030A. Before starting the experiment, the instrument was calibrated with protein standards G2025A supplied by Hewlett-Packard; mass calibration was used by peak centroiding at the 80% level. Sinnapinic acid(Hewlett-Packard) was used as a matrix source. Samples were prepared by spotting 1 uL of a mixture of protein, in phosphate buffer, with the matrix solution(1:1, v/v) on the gold surface of the probe with subsequent evaporation under vacuum. Ionization was accomplished with a laser radiating at a 337-nm wavelength(5 ns pulses, laser energy 1.97 uJ) in both single shot and multiple shot modes. The analyzer was used in the linear mode at an accelerating voltage of 28 kV. The obtained spectra represent the sum of consecutive laser shots and have not been smoothed.

**SDS-PAGE Analysis.** SDS-PAGE was used to monitor the preparation and purification of the EGF-Genistein conjugates. 10 - 20% tris tricine gradient gels (BioRad Laboratories) were stained with GelCode Blue to visualize the protein bands.

**Breast Cancer Cells.** MDA-MB-231 (ATCC HTB-26) is an EGF-R positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51 year old patient. BT-20 (ATCC HTB-19) is another EGF-R positive breast cancer cell line isolated from the primary breast tumor of a 74 year old patient with grade II mammary adenocarcinoma. SK-BR-3(ATCC HTB-30) is an adenocarcinoma of the mammary gland which was isolated from the pleural effusion of a 43 year old female; SQ-20B is a squamous cell carcinoma of the head and neck.

MDA-MB-231 cells are cultured in Leibovitz's L-15 medium plus glutamine; BT-20 breast cancer cells are maintained in MEM medium containing 0.1 mM NEAA and Earle's BSS; SK-BR-3 cells are cultured in McCoy's medium and SQ-20B in DMEM. All media are further supplemented with 10 % fetal bovine serum(DMEM contains 20% FBS, not heat-inactivated). For subculturing, medium is removed from the flasks containing a confluent layer of cells and fresh 0.25% trypsin added for 1-2 min. Trypsin is removed and cultures incubated for 5-10 min at 37°C until the cells detached. Fresh medium is then added and the cells aspirated and dispensed into new flasks.

**Cytotoxic Activity of EGF-Genistein and Related EGFCconjugates.** The specific cytotoxic activity of the EGF-Genistein conjugates is determined initially using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim Corp., Indianapolis, IN). Briefly, exponentially growing breast cancer cells are seeded into a 96-well plate at a density of  $2.0 \times 10^4$  cells/well and incubated for 18 - 24 hr at 37°C prior

to drug exposure. On the day of treatment, culture medium is carefully aspirated from the wells and replaced with fresh medium containing the EGF-Genistein conjugates or unconjugated EGF. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 48 - 72 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere( MDA-MB-231 cells are incubated in the absence of CO<sub>2</sub>). To each well, 10 µl of MTT (0.5 mg/ml final concentration) was added and the plates incubated at 37°C for 4 hours to allow MTT to form formazan crystals by reacting with metabolically active cells . The formazan crystals were solubilized for a minimum of 4 hr at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbance of each well is measured in a microplate reader (Labsystems) at 540 nm. The absorbance is a measure of cell viability; the greater the absorbance the greater the cell viability.

**Colony Assays.** After overnight treatment with EGF-Gen or PBS, cells were resuspended in clonogenic medium consisting of alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/mL/dish and cultured in a humidified 5% CO<sub>2</sub> incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as % inhibition of clonogenic cells at a particular concentration of the test agent using the formula: % Inhibition = (1 - Mean # of colonies [Test] / Mean # of colonies [Control]) x 100.

**Zebra Fish Embryo Test System.** Zebra fish embryos were incubated with EGF-Genistein conjugates and observed for inhibition of cell division and embryonic development. Dechorionated embryos, at the 2 - 4 cell stage, were exposed to the drugs in 24-well plates and incubated at a constant temperature of 82° F. Various concentrations of the conjugates were added to the embryonic medium in a total volume of 500 uL and the embryos observed for 30 minutes - 3 hr.

## RESULTS AND DISCUSSION

Our initial EGF-Genistein conjugates were formed using Sulfo-SANPAH as the photolabile crosslinker. EGF was modified using a 10:1 molar ratio of Sulfo-SANPAH : EGF followed by 60 minutes of photolysis in the presence of longwave UV and a 10 - 20-fold molar excess of Genistein. Size-exclusion HPLC revealed the presence of high-molecular weight material and SDS - PAGE showed the presence of EGF multimers. We also noted that this EGF conjugate precipitated out of solution during short-term storage at 4° C or when frozen for longer periods of time further reducing the yield of the active EGF - Gen conjugate.

Photolyzing the highly SANPAH-modified EGF at high protein concentrations appeared to be causing the formation of EGF-EGF multimers and denaturing the EGF so we carried out photolysis on the Sulfo-SANPAH-Genistein mixture(in DMSO) prior to the addition of the EGF. This "pre-photolysis" mixture contained a 10:1 or 2.5:1 molar excess of Genistein to crosslinker in order to increase the opportunity for the active nitrene to link to Genistein rather han to another SANPAH or EGF molecule. EGF was added to this mixture following photolysis and unreacted SANPAH and Genistein were removed using G-25 Sephadex column chromatography. A representative analytical size-exclusion HPLC analysis revealed the presence of high molecular weight aggregates eluting from 30 - 45 minutes post-injection (**Figure 1**). Unmodified EGF typically elutes in this system at 50 - 60 minutes.

Reverse-phase HPLC analysis was performed on EGF- Genistein conjugates prepared using a 4:1 ratio of the SANPAH crosslinker to EGF. **Figure 2A** shows the HPLC pattern for the SANPAH-modified EGF itself (in the absence of Genistein), **Figure 2B** shows the pattern for the EGF-Gen

conjugate formed when the SANPAH-modified EGF is photolyzed in the presence of a 10:1 molar ratio of Genistein, and **Figure 2C** shows the pattern for an EGF-Gen conjugate formed by photolyzing the SANPAH and Genistein prior to adding the EGF.

In this series of experiments, EGF had a retention time between 13 and 17 minutes and is detected at wavelengths of 220 and 280 nm. Since there is no detectable absorbance at 480 nm (characteristic of the SANPAH crosslinker), this peak represents unmodified EGF. The UV spectrum shows a peak at 280 nm which is characteristic of aromatic amino acid residues in proteins. All of the HPLC traces show a number of peaks which are detectable at 220, 280, and 480 nm. The UV spectra of these peaks reveal the absorbance peak at 280 nm (characteristic of EGF) as well as an absorbance at 480 nm indicating the presence of the SANPAH moiety.

This reverse-phase system was also used to verify the presence of unreacted EGF in fraction III from the size-exclusion HPLC separation shown as **Figure 1**. **Figure 2D** shows that HPLC fraction III has a retention time of 12.196 minutes and a UV spectrum characteristic of unmodified EGF.

When Genistein has been added to the conjugation mixture (**Figures 2B and 2C**), the presence of unreacted Genistein, with a retention time of 15 - 18 minutes, can be detected at wavelengths of 220 and 280 nm in this reverse-phase system. The UV spectrum is characteristic with a shoulder at 330 nm; UV spectra of potential EGF-Genisten conjugates, eluting at 36 - 38 minutes, possess this shoulder along with an absorbance at 480 nm.

An EGF-Genistein conjugate was made using a prephotolyzed mixture of SANPAH and Genistein with Genistein in a 10:1 molar excess. Photolysis was carried out for 48 hr under longwave UV and the mix added to EGF at a

2:1 molar ratio of crosslinker to EGF. This conjugate was put through the preparative size-exclusion HPLC and fractions collected for the MTT assay. In this experiment, the so-called "heavy material" in fractions I and III (**Figure 3A**) showed significant inhibition of BT-20 cells in the MTT assay, whereas fraction IV(unreacted EGF) and the unpurified mixture showed no inhibition (**Figure 3B**). It is possible that unmodified EGF possesses a greater affinity for the EGF receptor and could successfully block the binding of the EGF-Gen conjugate.

We also made EGF- 24 and EGF - 41 conjugates by prephotolyzing mixtures containing an excess of DDE24 or DDE41 to SANPAH. Lower ratios were used because these compounds are very insoluble in aqueous solutions. EGF was added after the photolysis but these conjugates were not significantly more effective at inhibiting breast cancer cells than the conjugates prepared by directly linking DDE24 or DDE41 in the absence of photolysis.

We then substituted shorter chain-length and less hydrophobic crosslinkers for SANPAH in order to reduce aggregation due to protein-protein hydrophobic interactions. The short-chain crosslinker, ANB-NOS, results in less precipitation/aggregation than was seen using Sulfo-SANPAH. Since Genistein is relatively insoluble in aqueous solutions, we carried out the pre-photolysis using a 5:1 or 10:1 molar ratio of Genistein to crosslinker and a 5:1 or 10:1 ratio of crosslinker:EGF. EGF-Gen conjugates were prepared by photolyzing ANB-NOS-modified EGF for one hr under longwave UV in the presence of excess Genistein or by prephotolyzing the ANB-NOS-Genistein mixture for 3.25 or 6.25 hr under longwave UV before adding EGF. The final DMSO concentration was maintained at 10%. These conjugates were subsequently tested at concentrations of 25 and 50 ug/mL for their effects on zebra fish embryo

cell division and development. EGF was included in these experiments as a control.

Only the EGF-Gen conjugates made by prephotolyzing the ANB-NOS/Genistein mixture for 6.25 hr showed an effect on embryogenesis. A 10:1 ratio of the ANB-NOS crosslinker to EGF was used; the conjugate containing a 5:1 ratio of Genistein to ANB-NOS in the prephotolysis mix caused lysis of the embryos after one hr incubation at both concentrations. The conjugate prepared with a 10:1 ratio of Genistein in the prephotolysis mix also caused lysis of the embryos within one hr of incubation at the high concentration but required up to two hr to see the same affect at the low concentration. The EGF control showed normal embryo development. **Figure 4** shows representative results of this assay.

We then obtained the SQ-20B and SK-BR-3 cell lines and used them to test a variety of EGF conjugates, including EGF- Gen prepared using the SFAD crosslinker. SFAD-modified EGF was photolyzed in the presence of excess Genistein and SFAD/Genistein mixtures were prephotolyzed for various periods of time using a mid-range UV lamp. We also linked Genistein and P97 (a rationally designed small molecule EGFR inhibitor developed at the Hughes Institute) to EGF using longwave UV and photolysis in the presence of SANPAH-modified EGF. Additional EGF conjugates were prepared from DDE24 and DDE41, either by direct linkage or using ANB-NOS as the crosslinker. **Figures 5A - 5D** show results of MTT assays using these EGF conjugates against these new cell lines. All of the conjugates, as well as the EGF control, exhibit some degree of inhibition of these cell lines indicating that breast cancer cell lines vary in their susceptibility to the EGF conjugates.

## **SECTION II. ANIMAL STUDIES**

### **MATERIALS AND METHODS**

The detailed procedures for murine and primate toxicity studies were detailed in the original grant application and also reported in the previously submitted manuscripts regarding the animal toxicity of the first generation EGF conjugates.

### **RESULTS AND DISCUSSION**

**I. Toxicity Studies in Monkeys.** In our last report, we mentioned that we examined the toxicity of EGF-ANB-NOS-Genistein and EGF-ANB-NOS-DDE41 (EGF-41) in cynomolgus monkeys. Both agents were well tolerated by monkeys. A detailed report of the clinical findings and raw data was enclosed as **Appendix 2** in the last report. The monkeys have been sacrificed and a detailed histopathology report is included in the present report as **Appendix 2**. No evidence of test article-related lesions was found in monkey 68-K treated with a 1 mg i.v. bolus of EGF-41; 68-I treated with a 5 mg i.v. bolus of EGF-41; 68-N treated with a 1 mg i.v. bolus of EGF-ANB-NOS-Genistein; or 68-J treated with a 5 mg i.v. bolus of EGF-ANB-NOS-Genistein.

**II. Toxicity Studies in SCID Mice.** We examined in a small pilot study the toxicity of combined chemo-biotherapy regimens employing EGF-Genistein plus cytoxan, taxol, methotrexate, or adriamycin in healthy SCID mice. Taxol + EGF-Genistein, Cytoxan + EGF-Genistein as well as Methotrexate + EGF-Genistein combinations were well tolerated. The experimental data are included in **Appendix 3**. More extensive toxicity studies will be performed during the next grant period.

**III. Efficacy Studies in SCID Mice.** We examined the biologic activity of various chemo-biotherapy regimens in SCID mice xenografted with MDA-MB-231 human breast cancer cells. These regimens utilized EGF-Genistein at a high dose level ( $100 \mu\text{g}/\text{mouse} = 5 \text{ mg/kg}$ ) and a 4-day treatment schedule (appendix 4). While the combination therapies showed significant anti-cancer activity, no additional benefit was achieved by the combination with EGF-Genistein.

During the next grant period, we will continue our stepwise preclinical development of EGF-Genistein conjugates as a potential new class of anti-breast cancer drugs. The studies will focus both on the conjugation chemistry of novel EGFR tyrosine kinase inhibitors as well as the evaluation of their toxicity, pharmacokinetics, and efficacy in established preclinical animal models as in the previous years.

## **Appendix I**

## **Figure Legends**

**Figure 1-** Figure 1 shows an example of a size-exclusion HPLC profile of an EGF-Genistein conjugate prepared using a 4:1 ratio of crosslinker to EGF and a prephotolyzed mixture containing a 10:1 molar excess of Genistein to SANPAH. The Beckman System Gold HPLC was equipped with a TSKG3000PW analytical column equilibrated in 100 mM sodium phosphate buffer, pH 6.8, at a flow rate of 0.2 mL/minute. Fractions are labeled I, II, and III.

**Figure 2A -** Figure 2A shows a reverse-phase HPLC pattern of EGF-SANPAH made using a 4:1 molar ratio of SANPAH to EGF. UV spectra are included for the major peaks; unmodified EGF elutes at 16.864 minutes in this run. The spectrum of the peak eluting at 30.026 is characteristic of the SANPAH crosslinker.

**Figure 2B -** Figure 2B shows a reverse-phase HPLC pattern of EGF-Genistein made by photolyzing the EGF-SANPAH in the presence of a 10-fold molar excess of Genistein. In addition to the unmodified EGF(retention time of 16.858 min), a peak of unreacted Genistein is also present at 18.042 min. UV spectra are included for representative peaks; the peak eluting at 37.474 min. appears to have characteristics of EGF, SANPAH, and Genistein.

**Figure 2C -** Figure 2C shows a reverse-phase HPLC pattern of EGF-Genistein made by prephotolyzing the SANPAH/Genistein mixture prior to adding the EGF. The pattern shows peaks characteristic of unmodified EGF and Genistein, as well as of a possible EGF-Genistein conjugate.

**Figure 2D -** Figure 2D is a reverse-phase HPLC trace of fraction III shown in Figure 1 . The peak with a retention time of 12.196 min. and a UV spectrum characteristic of unmodified EGF verifies that the size-exclusion chromatography is able to remove a significant amount of the free EGF

remaining in the conjugation mixture.

**Figure 3A** shows a preparative size-exclusion purification of an EGF-Genistein conjugate prepared using the prephotolyzed SANPAH/Genistein mixture. Fractions were isolated and tested against the BT-20 breast cancer cell line using the MTT assay(**Figure 3B** ). In the MTT assay, the greater the response(y-axis), the greater the cell viability.

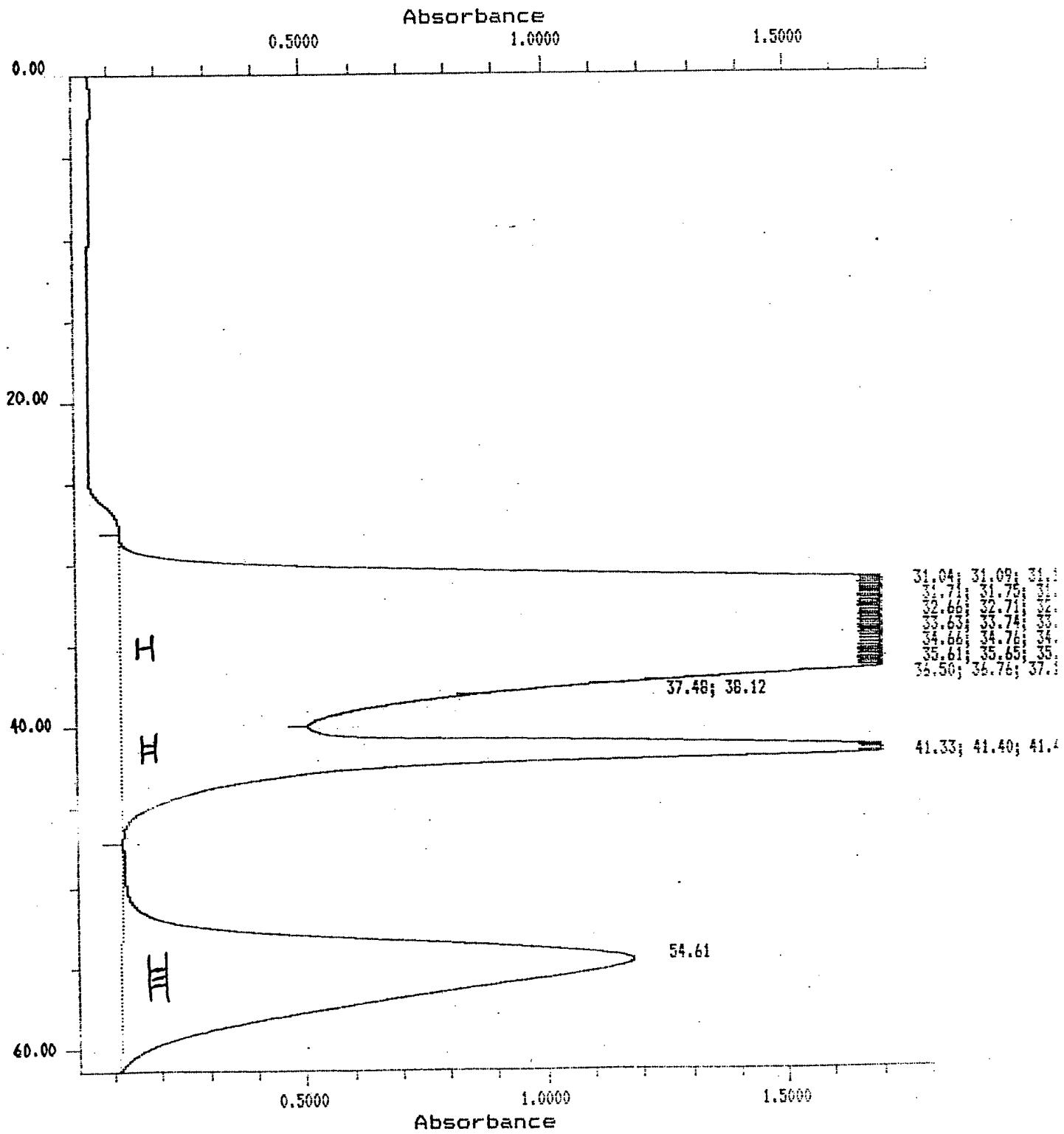
**Figure 4A** shows a zebra fish embryo treated with 50 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 5:1 ratio of Genistein to ANB-NOS. Cell lysis is evident after one hr of incubation.

**Figure 4B** shows zebra fish embryos treated with 25 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 10:1 ratio of Genistein to ANB-NOS. Cell lysis is present here as well.

**Figure 4C** Zebra fish embryo showing normal development.  
EGF-Genistein conjugates prepared using the ANB-NOS crosslinker at a 1:10

**Figures 5A - 5D** - MTT assays using SQ-20B and SK-BR-3 cell lines. EGF alone was tested as well as various EGF-Gen conjugates (Fig. 5A - EGF-SANPAH-Genistein, Fig. 5B - EGF-SFAD-Genistein), EGF-SANPAH-P97 (Fig. 5A), and EGF-24 conjugates (Fig. 5C and 5D). The EGF/24 conjugate was formed by direct linkage of DDE24 to EGF (i.e. no crosslinker was used). EGF/ANBNOS-24 was made by prephotolyzing DDE24 and the ANBNOS crosslinker. NPP in the figures means not prephotolyzed; EGF was modified and subsequently photolyzed for one hr in the presence of Genistein or P97.

**Figure 1-** Figure 1 shows an example of a size-exclusion HPLC profile of an EGF-Genistein conjugate prepared using a 4:1 ratio of crosslinker to EGF and a prephotolyzed mixture containing a 10:1 molar excess of Genistein to SANPAH. The Beckman System Gold HPLC was equipped with a TSKG3000PW analytical column equilibrated in 100 mM sodium phosphate buffer, pH 6.8, at a flow rate of 0.2 mL/minute. Fractions are labeled I, II, and III.



EGF/SAN (1:4), after PD-10 & NAP-10 purification. Reaction 4, 5/11/99.  
 A: H<sub>2</sub>O, 0.1% TFA. D: 80% ACN, 20% H<sub>2</sub>O, 0.1% TFA.  
 Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;  
 t=20, 43.5 % D; t=35, 100% D; t=50, 100% D, t=55, 20% D  
 ; t=56, stop. Flow=1.0 mL/min.

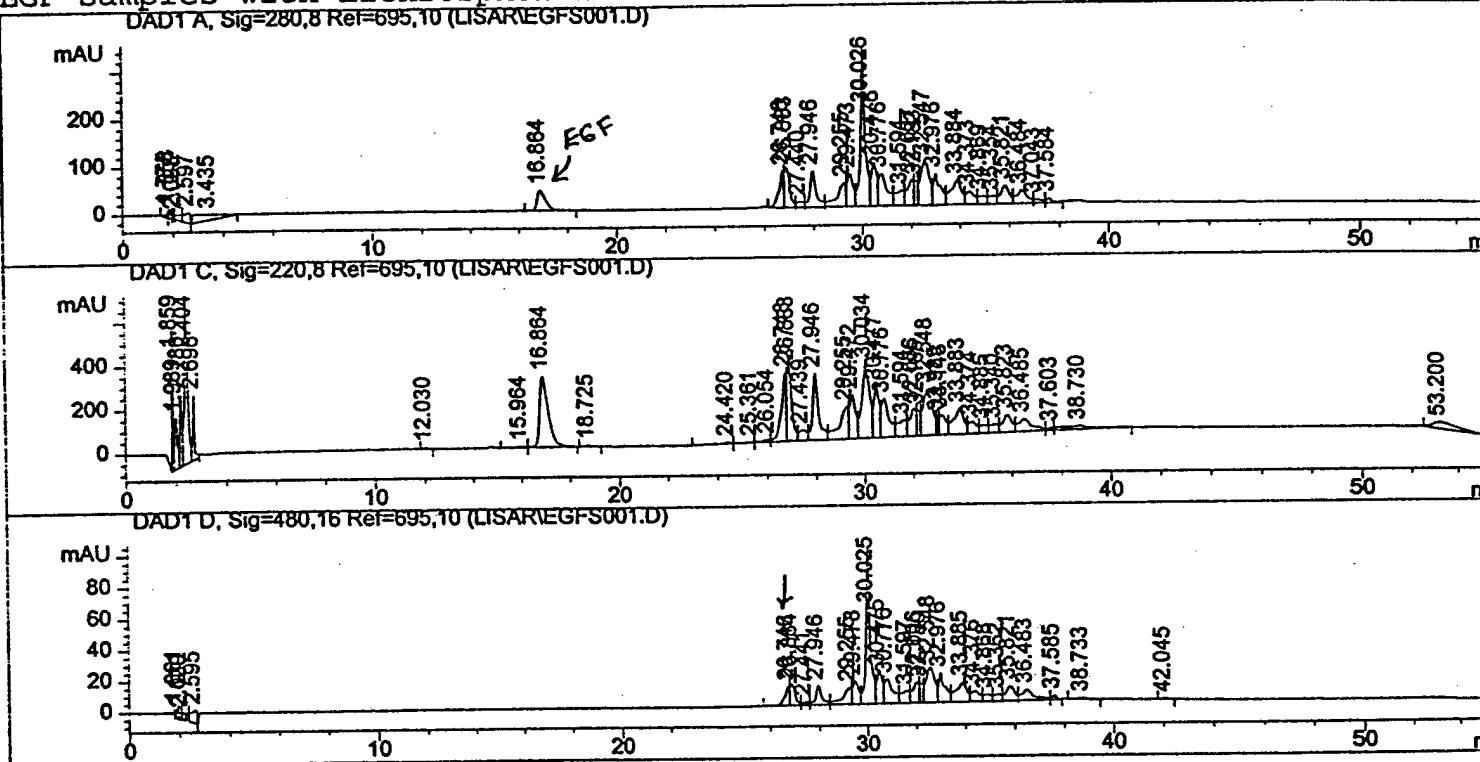
=====

Injection Date : 5/14/99 6:53:11 PM	Seq. Line : 2
Sample Name : EGF/SAN, #4	Vial : 3
Acq. Operator : L. Kuehn	Inj : 1
	Inj Volume : 50 $\mu$ l

Method : C:\HPCHEM\1\METHODS\LISAEGF2.M

Last changed : 5/14/99 4:15:43 PM by L. Kuehn

EGF samples with Lichrospher 100 column.



**Figure 2A** - Figure 2A shows a reverse-phase HPLC pattern of EGF-SANPAH made using a 4:1 molar ratio of SANPAH to EGF. UV spectra are included for the major peaks; unmodified EGF elutes at 16.864 minutes in this run. The spectrum of the peak eluting at 30.026 is characteristic of the SANPAH crosslinker.

Print of window 39: UV Apex spectrum of Peak 16.864 of EGFS001.D

UV Apex spectrum of Peak 16.864 of EGFS001.D  
DAD1, 16.867 (297 mAU, -) Ref=16.680 & 16.980 of EGFS001.D

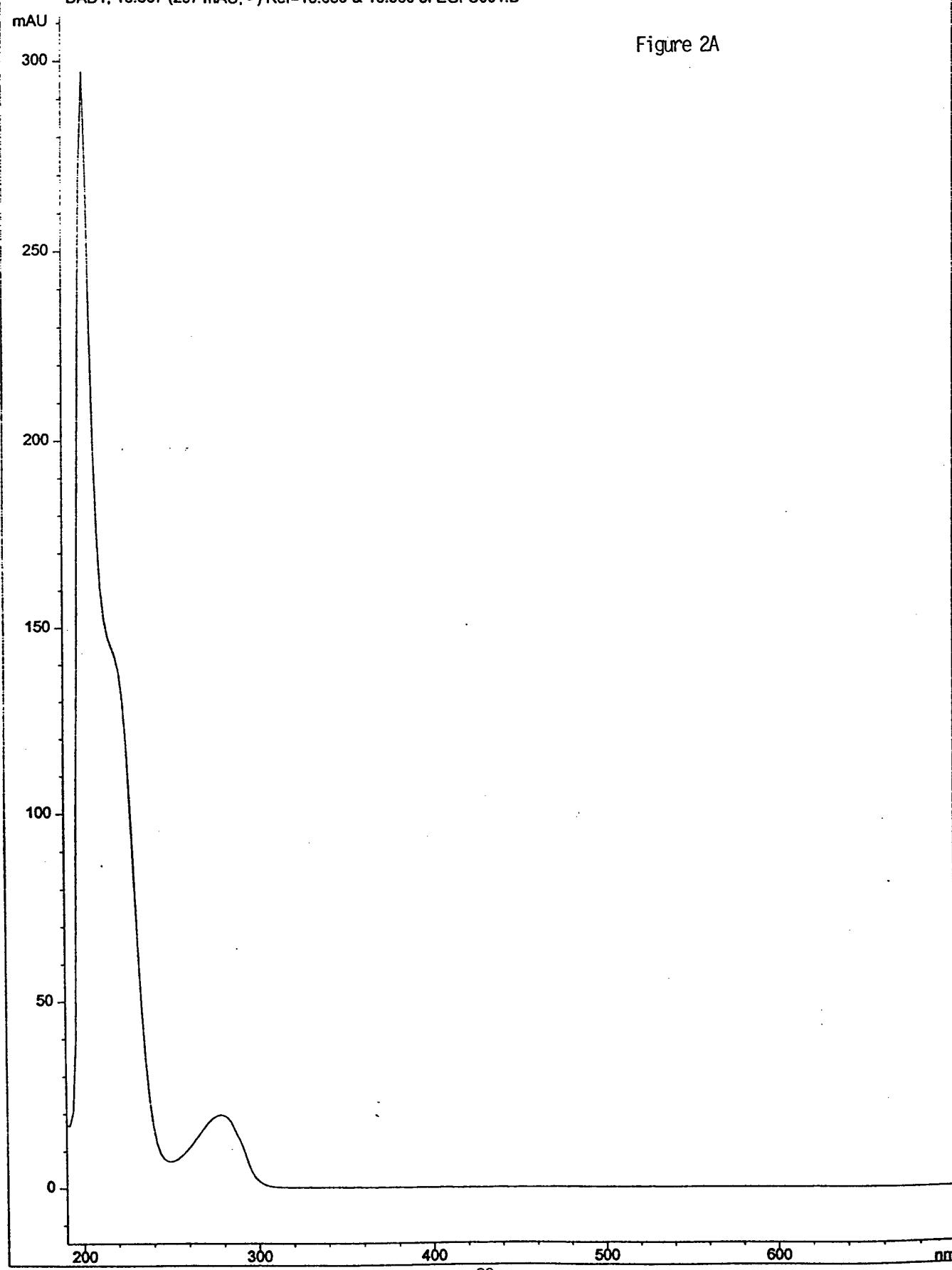


Figure 2A

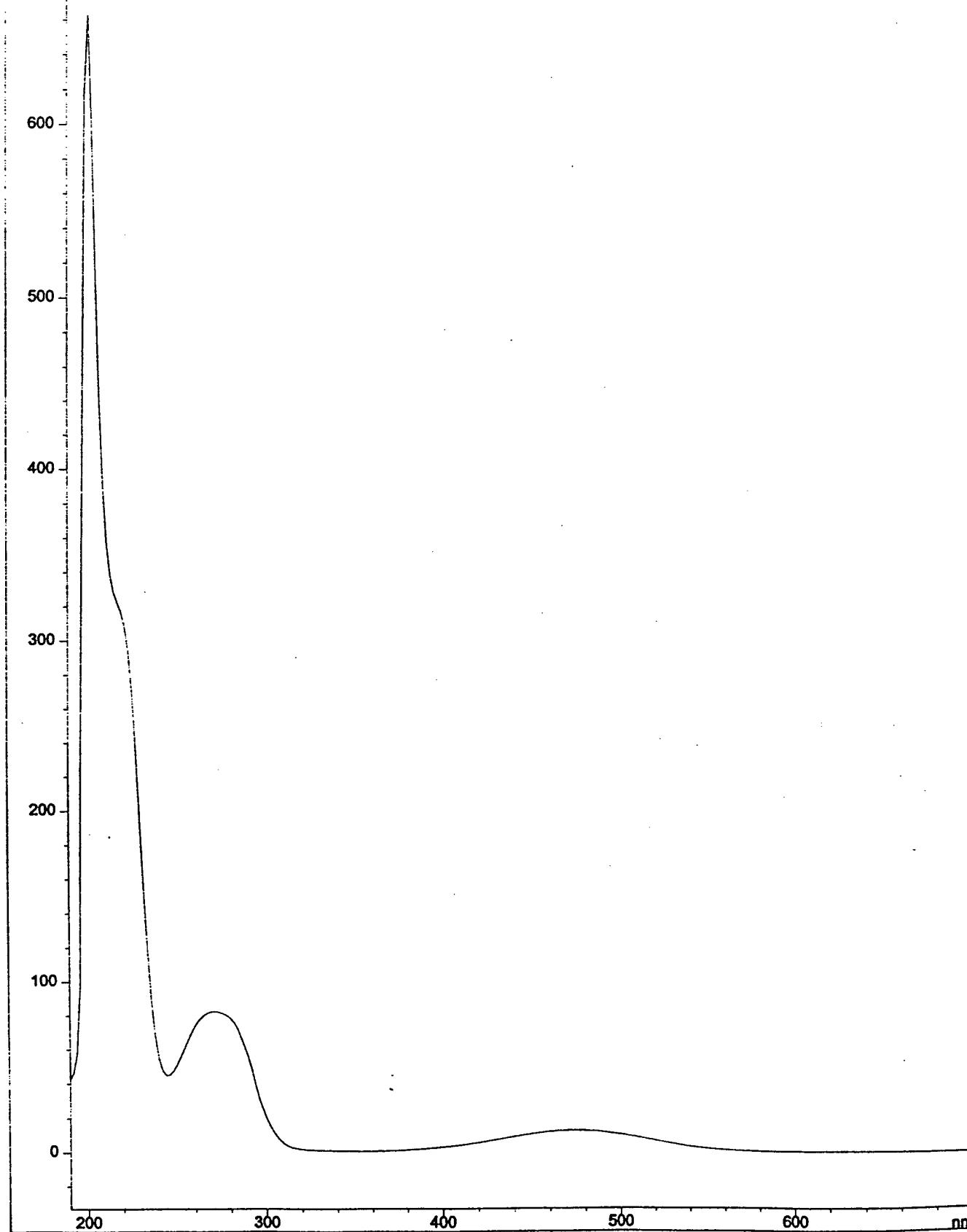
Print of window 39: UV Apex spectrum of Peak 26.883 of EGFS001.D

UV Apex spectrum of Peak 26.883 of EGFS001.D

\*DAD1, 26.880 (663 mAU, -) Ref=26.280 & 28.274 of EGFS001.D

mAU

Figure 2A



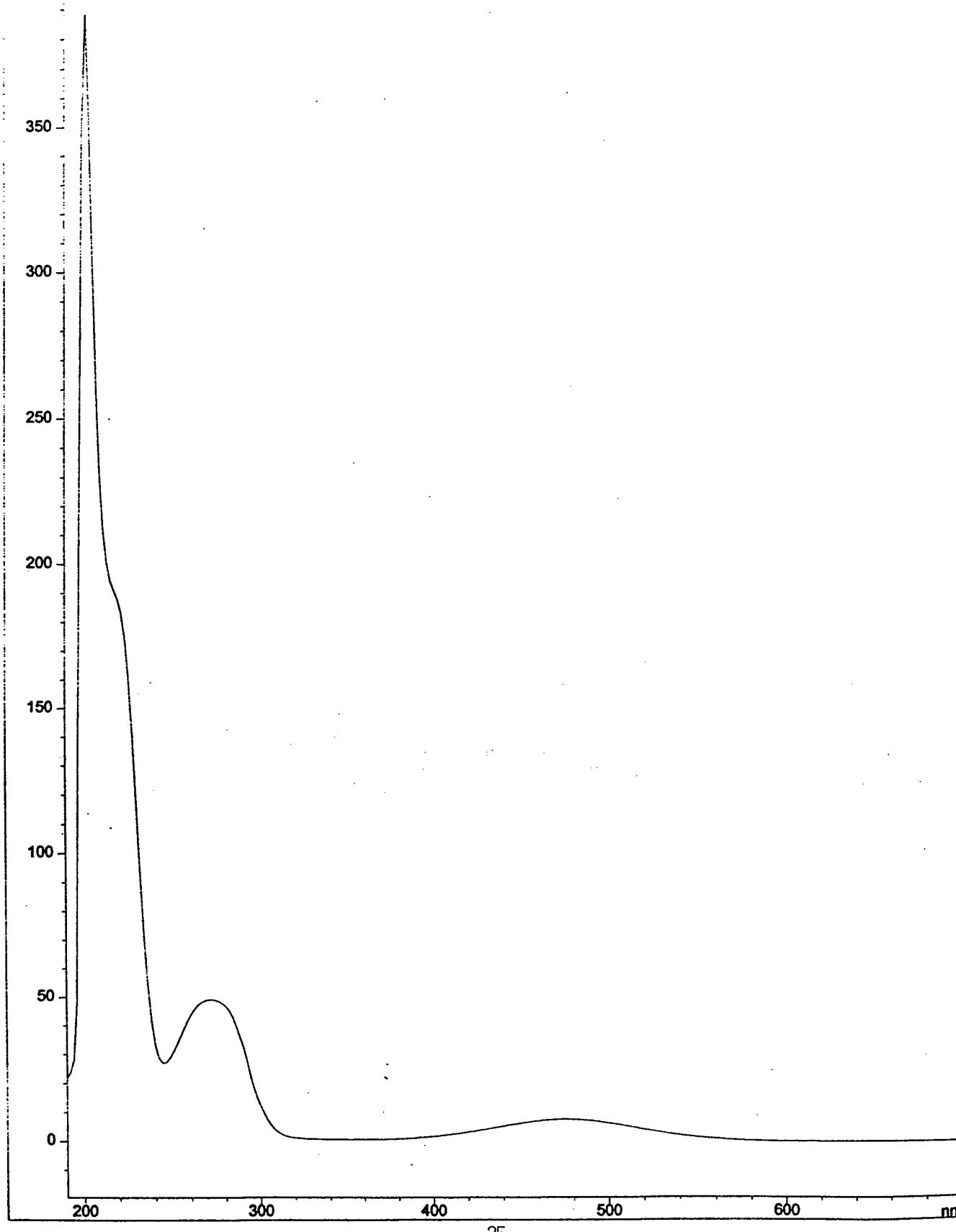
int of window 39: UV Apex spectrum of Peak 27.946 of EGFS001.D

UV Apex spectrum of Peak 27.946 of EGFS001.D

DAD1, 27.947 (388 mAU, -) Ref=27.814 & 28.074 of EGFS001.D

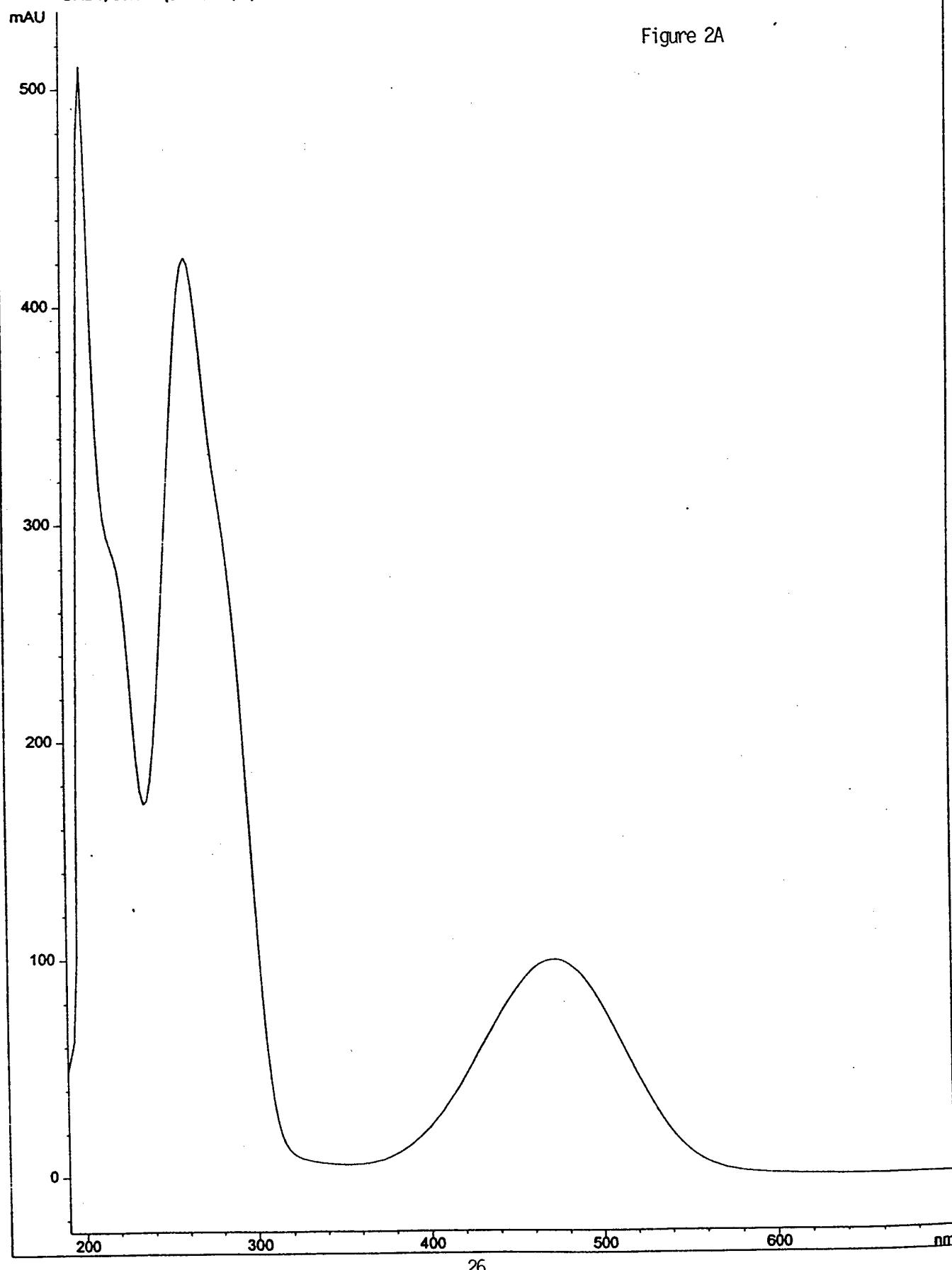
mAU  
400

Figure 2A



rint of window 39: UV Apex spectrum of Peak 30.026 of EGFS001.D

UV Apex spectrum of Peak 30.026 of EGFS001.D  
DAD1, 30.027(511 mAU, -) Ref=28.874 & 31.154 of EGFS001.D



Print of window 39: UV Apex spectrum of Peak 32.548 of EGFS001.D

UV Apex spectrum of Peak 32.548 of EGFS001.D

DAD1, 32.547 (346 mAU, -) Ref=31.154 & 33.480 of EGFS001.D

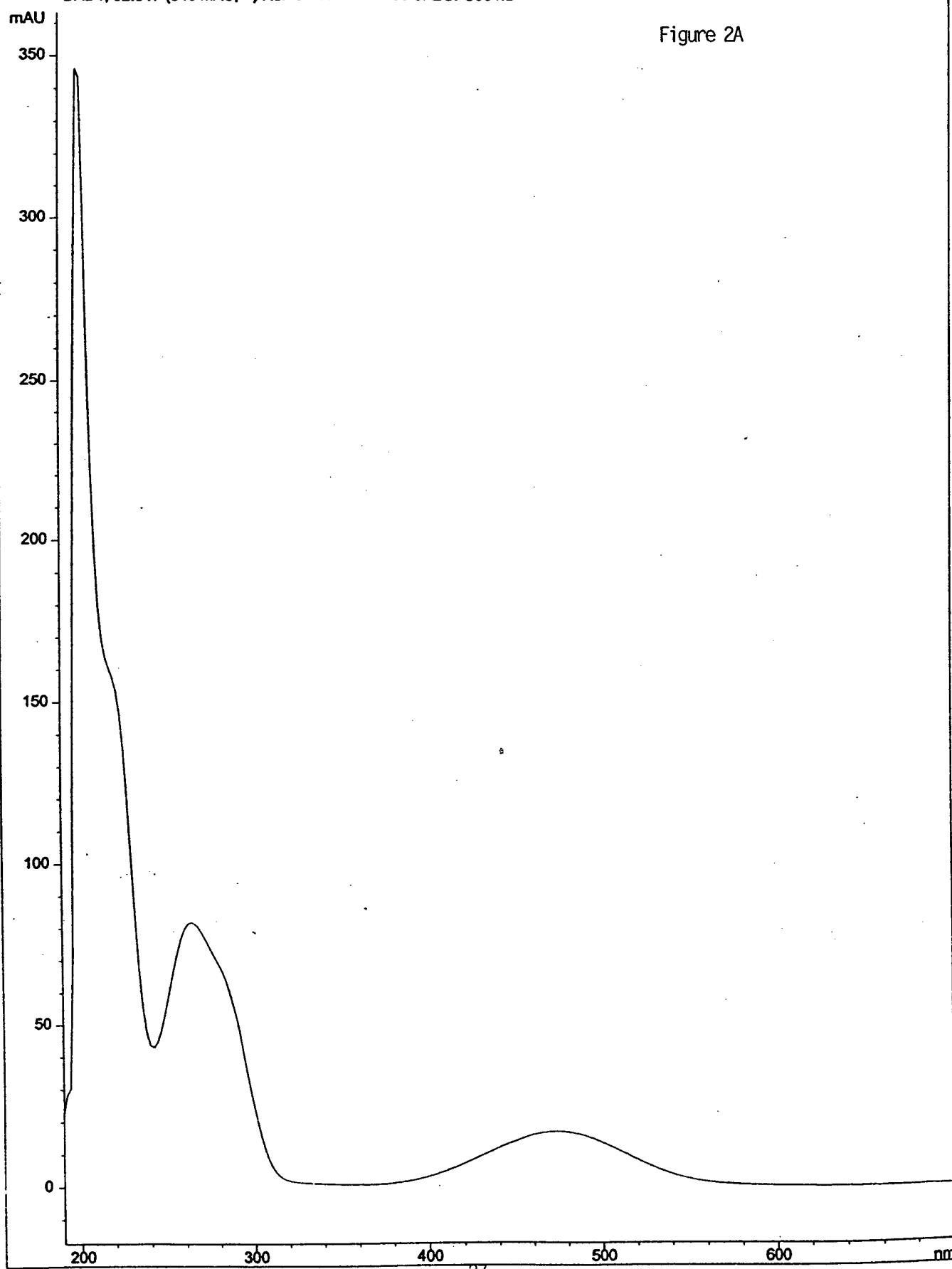


Figure 2A

Sample Name: EGF/SAN/Gen, #

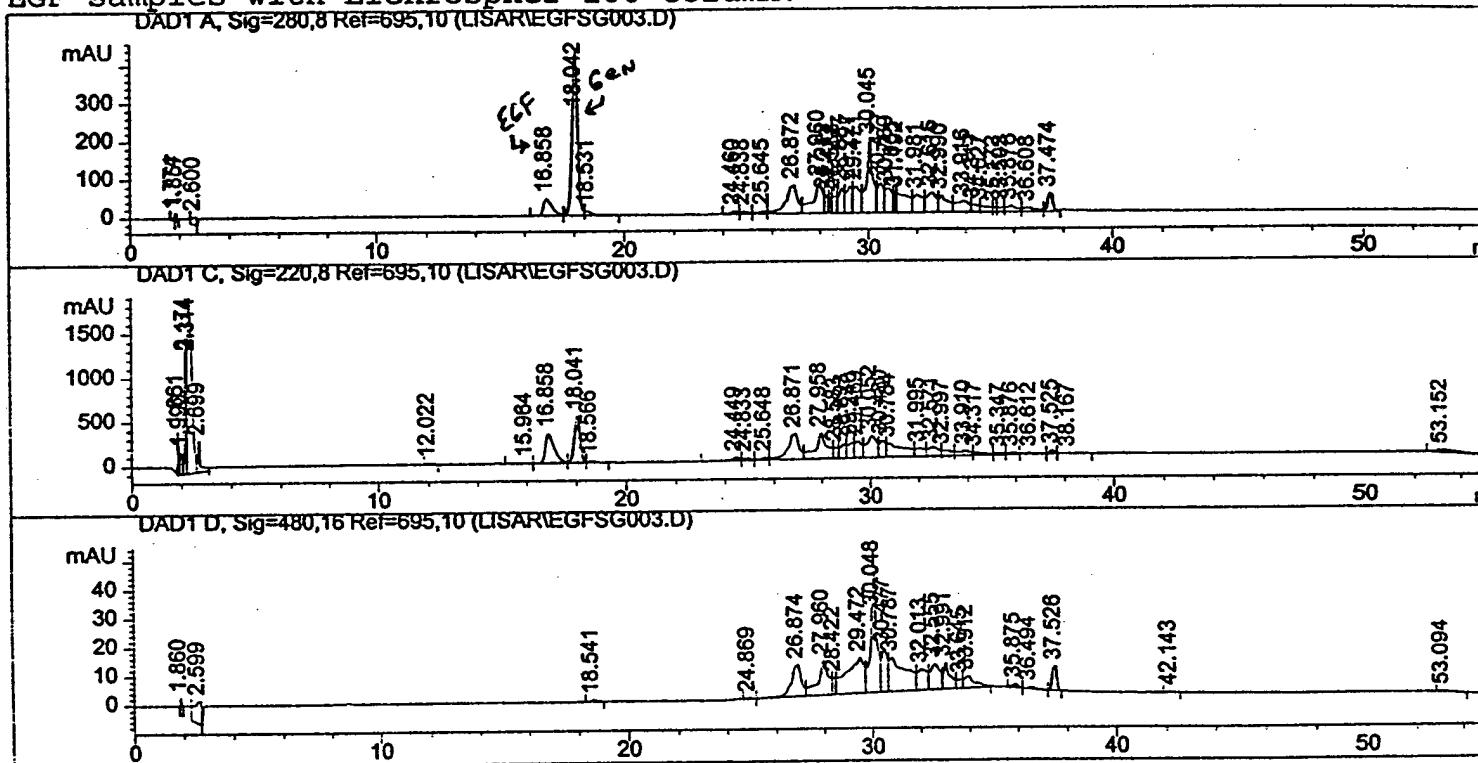
EGF/SAN-Gen (1:4) (1:10), not pp. Reaction 4, 5/12/99. 1mLWUV  
A: H<sub>2</sub>O, 0.1% TFA. D: 80% ACN, 20% H<sub>2</sub>O, 0.1% TFA.  
Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;  
t=20, 43.5 % D; t=35, 100% D; t=50, 100% D, t=55, 20% D  
; t=56, stop. Flow=1.0 mL/min.

=====  
Injection Date : 5/14/99 8:00:34 PM Seq. Line : 3  
Sample Name : EGF/SAN/Gen, #4 Vial : 4  
Acq. Operator : L. Kuehn Inj : 1  
Inj Volume : 50  $\mu$ l

Method : C:\HPCHEM\1\METHODS\LISAEGF2.M

Last changed : 5/14/99 4:15:43 PM by L. Kuehn

EGF samples with Lichrospher 100 column.



**Figure 2B** - Figure 2B shows a reverse-phase HPLC pattern of EGF-Genistein made by photolyzing the EGF-SANPAH in the presence of a 10-fold molar excess of Genistein. In addition to the unmodified EGF (retention time of 16.858 min), a peak of unreacted Genistein is also present at 18.042 min. UV spectra are included for representative peaks; the peak eluting at 37.474 min. appears to have characteristics of EGF, SANPAH, and Genistein.

int of window 39: UV Apex spectrum of Peak 18.042 of EGFSG003.D

UV Apex spectrum of Peak 18.042 of EGFSG003.D

\*DAD1, 18.044 (568 mAU, -) Ref=17.924 & 18.157 of EGFSG003.D

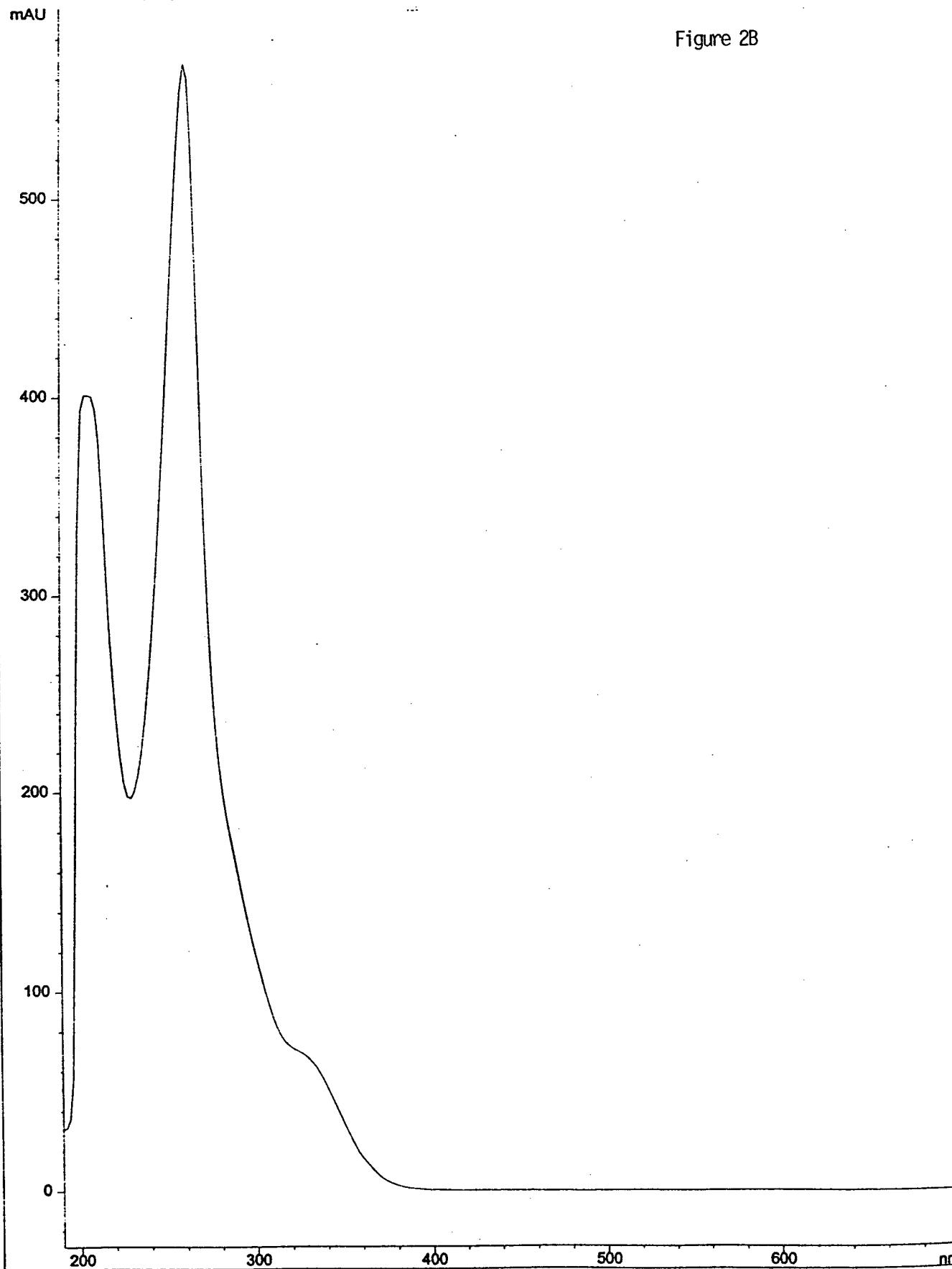


Figure 2B

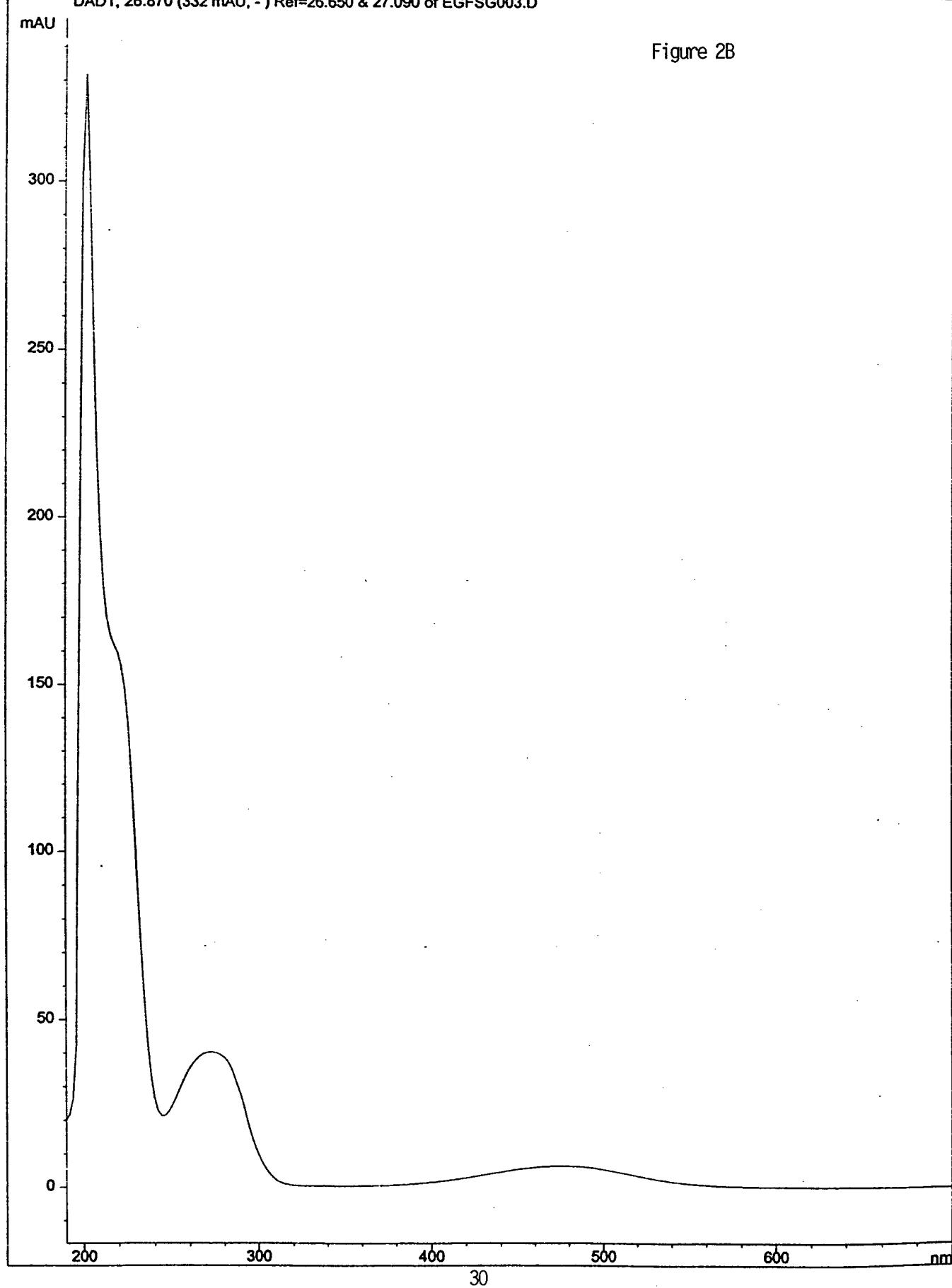
Print of window 39: UV Apex spectrum of Peak 26.872 of EGFSG003.D

UV Apex spectrum of Peak 26.872 of EGFSG003.D

DAD1, 26.870(332 mAU, -) Ref=26.650 & 27.090 of EGFSG003.D

mAU

Figure 2B



Print of window 39: UV Apex spectrum of Peak 27.958 of EGFSG003.D

UV Apex spectrum of Peak 27.958 of EGFSG003.D

DAD1, 27.957 (352 mAU, -) Ref=27.737 & 28.184 of EGFSG003.D

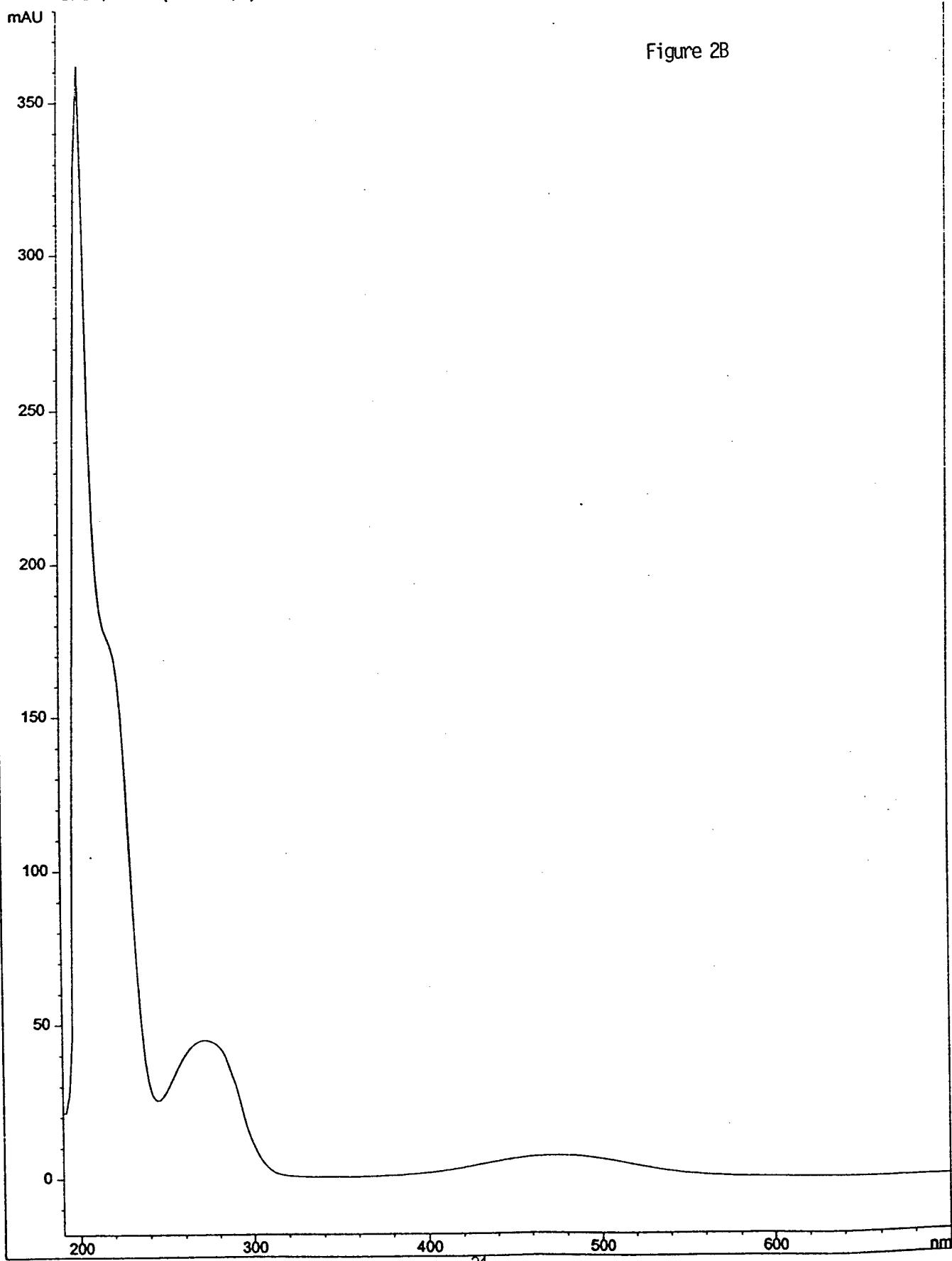


Figure 2B

rint of window 39: UV Apex spectrum of Peak 30.045 of EGFSG003.D

UV Apex spectrum of Peak 30.045 of EGFSG003.D

DAD1, 30.045 (144 mAU, -) Ref=29.924 & 30.170 of EGFSG003.D

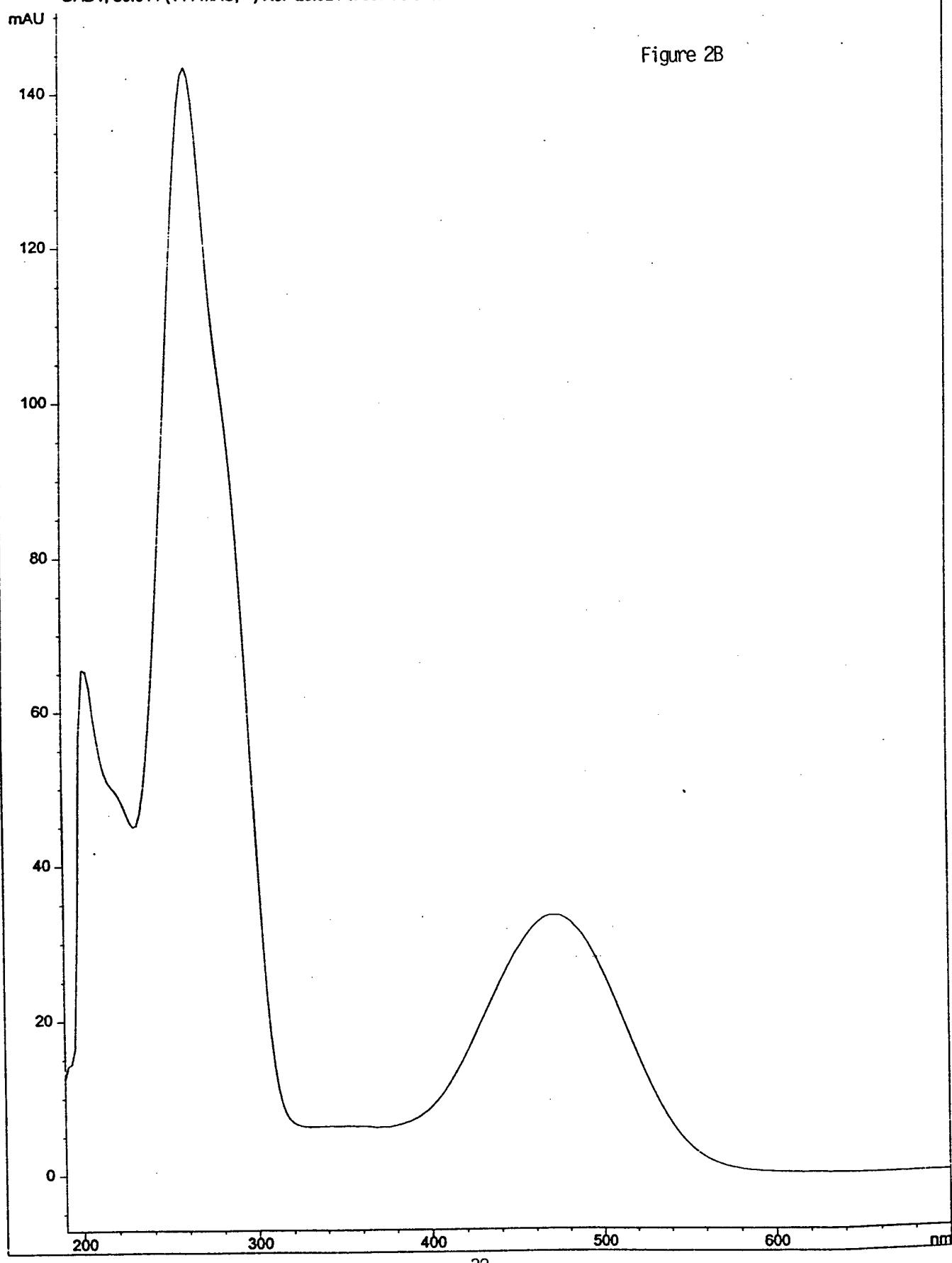
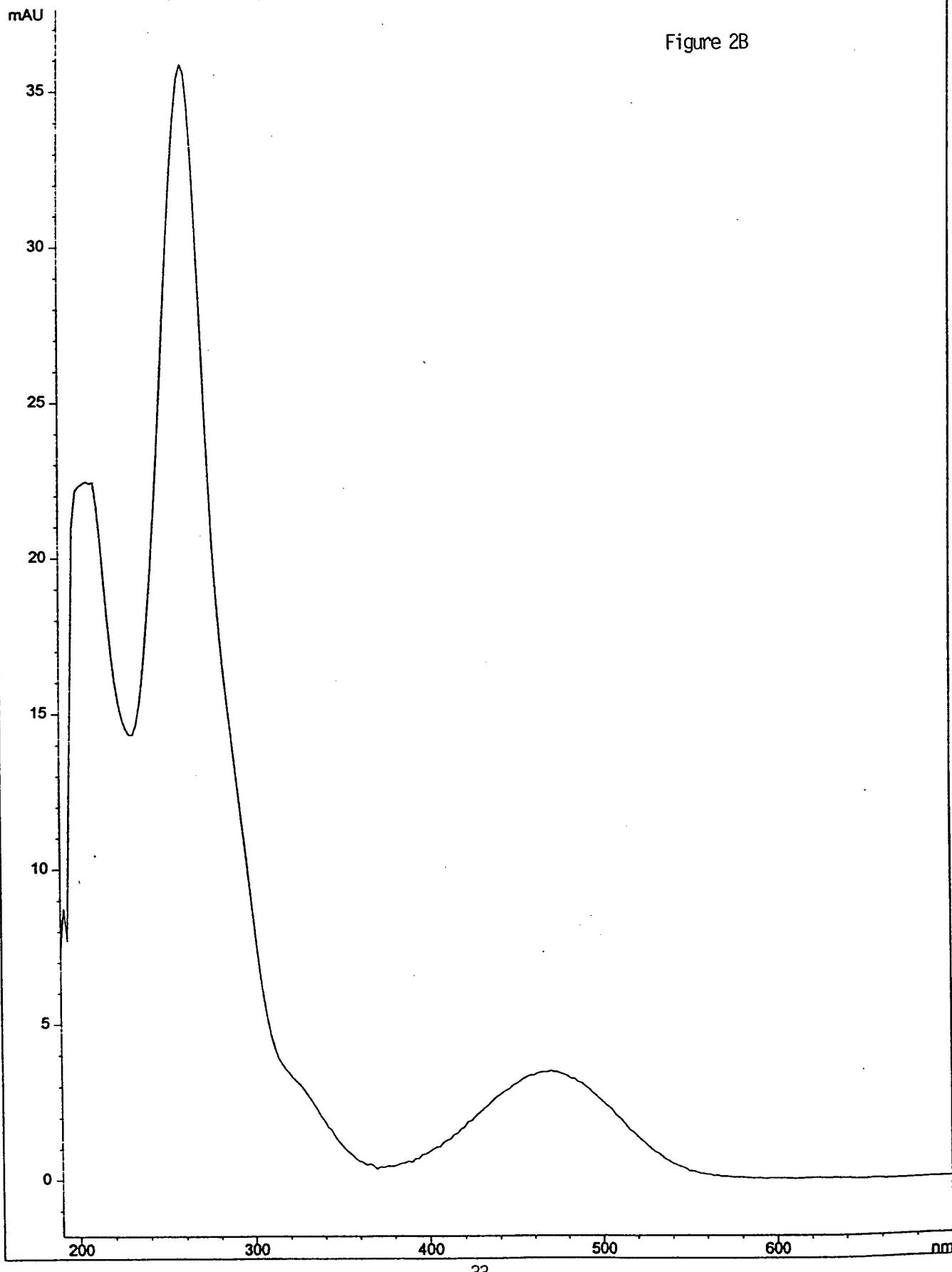


Figure 2B

rint of window 39: UV Apex spectrum of Peak 37.474 of EGFSG003.D

UV Apex spectrum of Peak 37.474 of EGFSG003.D

\*DAD1, 37.477 (35.9 mAU, -) Ref=37.377 & 37.570 of EGFSG003.D



EGF/SAN-Gen 1:4, 1:10. 16.5 hr LWUV. 5 mg/mL original  
1. pre-photolyzed

5/19/99. Parent compound

A: H<sub>2</sub>O, 0.1%TFA, 0.1%TEA. D: 80% ACN, 20% H<sub>2</sub>O, 0.1% TFA.

Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;  
t=20, 43.5% D; t=35, 100% D; t=50, 100% D; t=55, 20% D;  
t=56, stop. Flow = 1 mL/min.

=====

Injection Date : 5/20/99 6:30:54 AM Seq. Line : 10  
 Sample Name : EGF/SAN-Gen p Vial : 13  
 Acq. Operator : Lisa Kuehn Inj : 1  
 Inj Volume : 50  $\mu$ l

Acq. Method : C:\HPCHEM\1\METHODS\LISAEGF.M  
 Last changed : 5/19/99 4:21:34 PM by Lisa Kuehn  
 Analysis Method : C:\HPCHEM\1\METHODS\LISAGEN3.M  
 Last changed : 5/11/99 2:46:56 PM by L. Kuehn  
 Gen/SANPAH

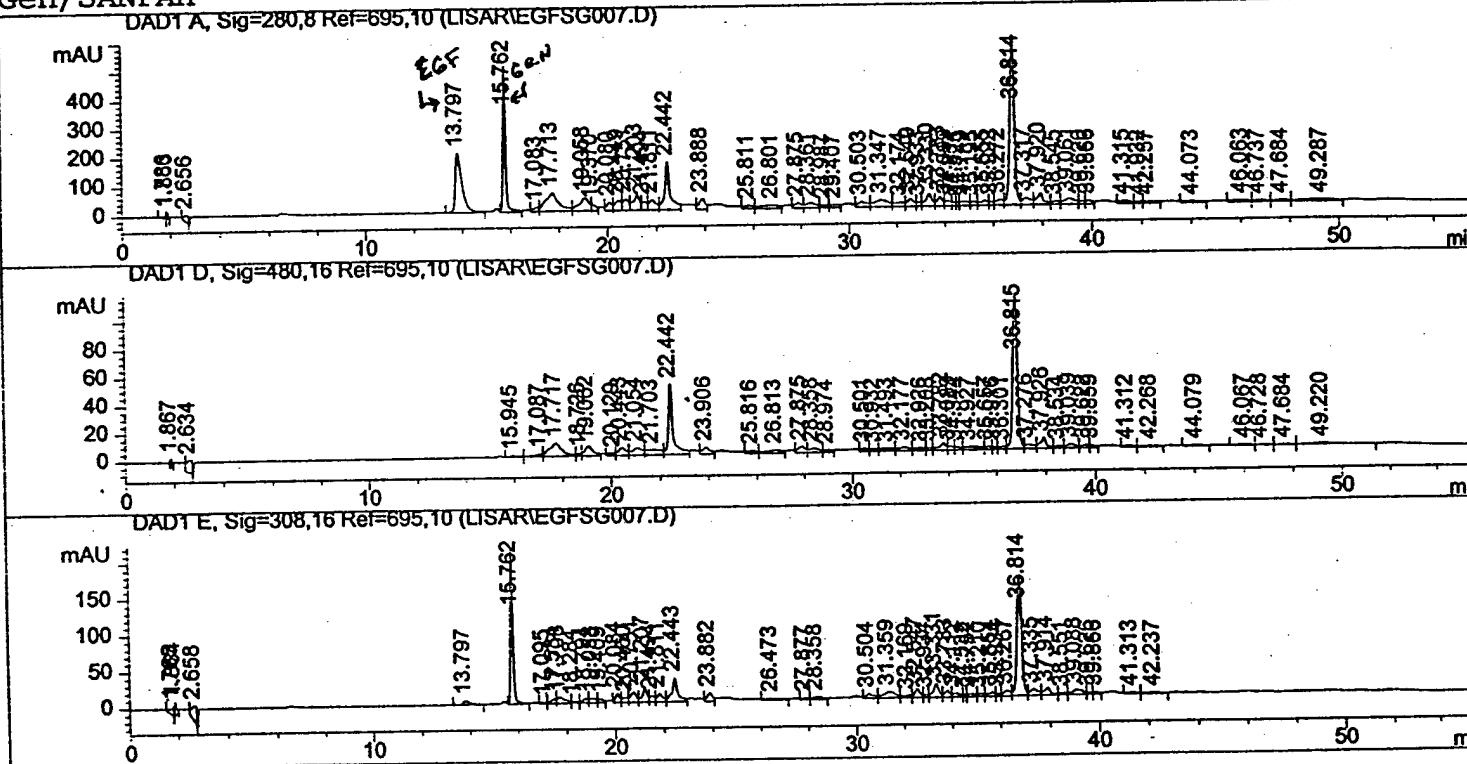


Figure 2C - Figure 2C shows a reverse-phase HPLC pattern of EGF-Genistein made by prephotolyzing the SANPAH/Genistein mixture prior to adding the EGF. The pattern shows peaks characteristic of unmodified EGF and Genistein, as well as of a possible EGF-Genistein conjugate.

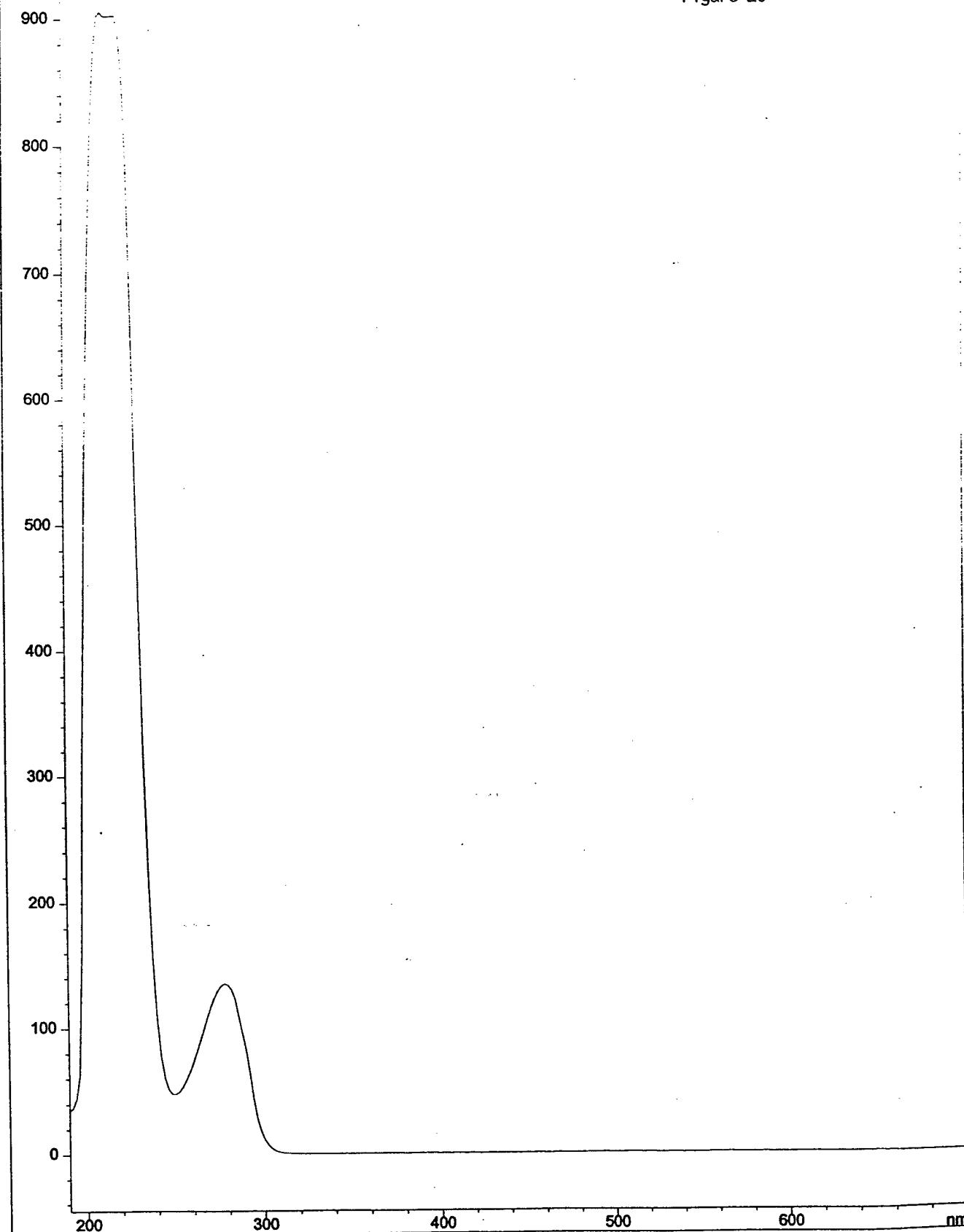
int of window 39: UV Apex spectrum of Peak 13.797 of EGFSG007.D

UV Apex spectrum of Peak 13.797 of EGFSG007.D

\*DAD1, 13.794 (905 mAU, -) Ref=13.647 & 13.947 of EGFSG007.D

mAU

Figure 2C

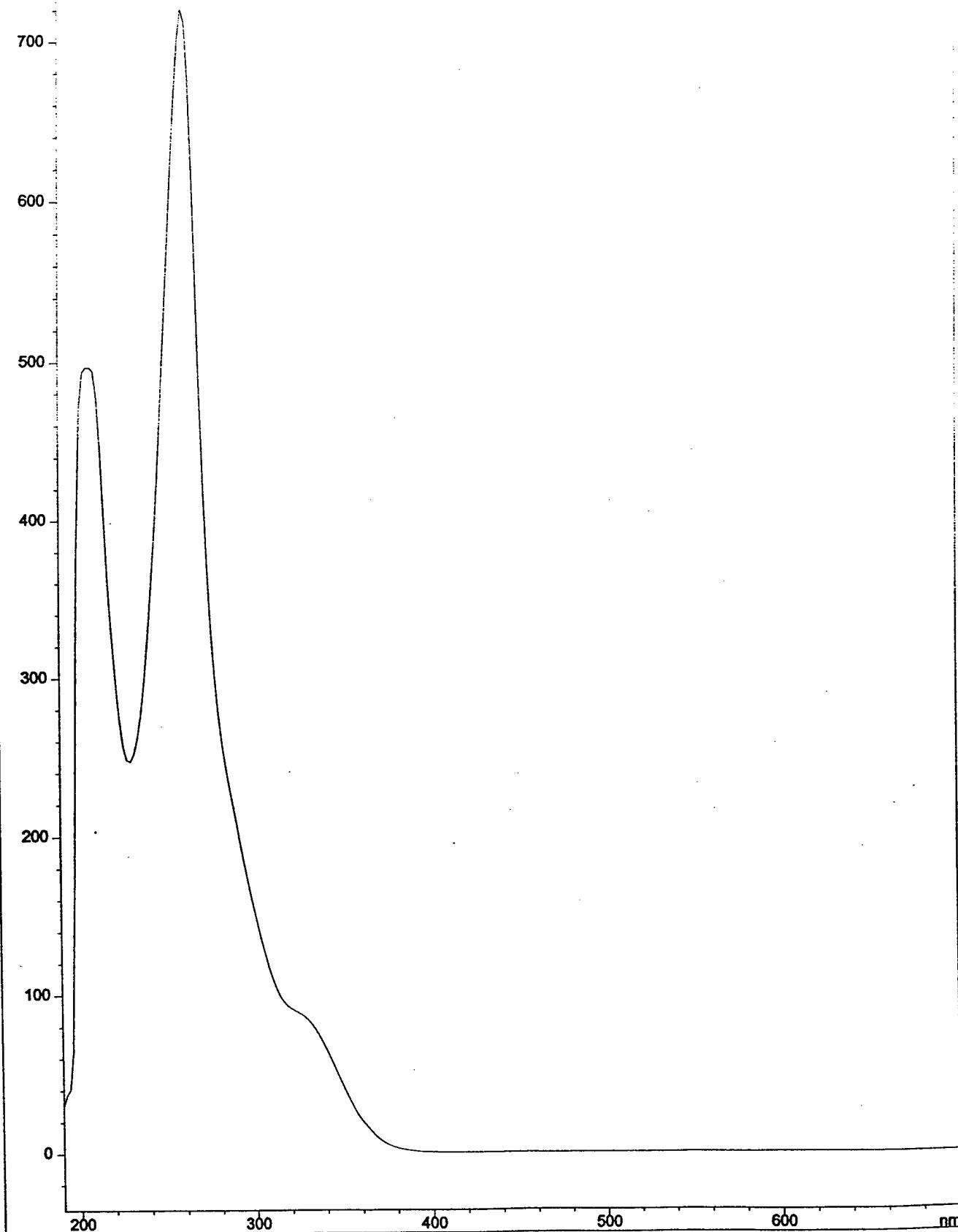


UV Apex spectrum of Peak 15.762 of EGFSG007.D

DAD1, 15.760(721 mAU, -) Ref=15.700 & 15.820 of EGFSG007.D

mAU

Figure 2C



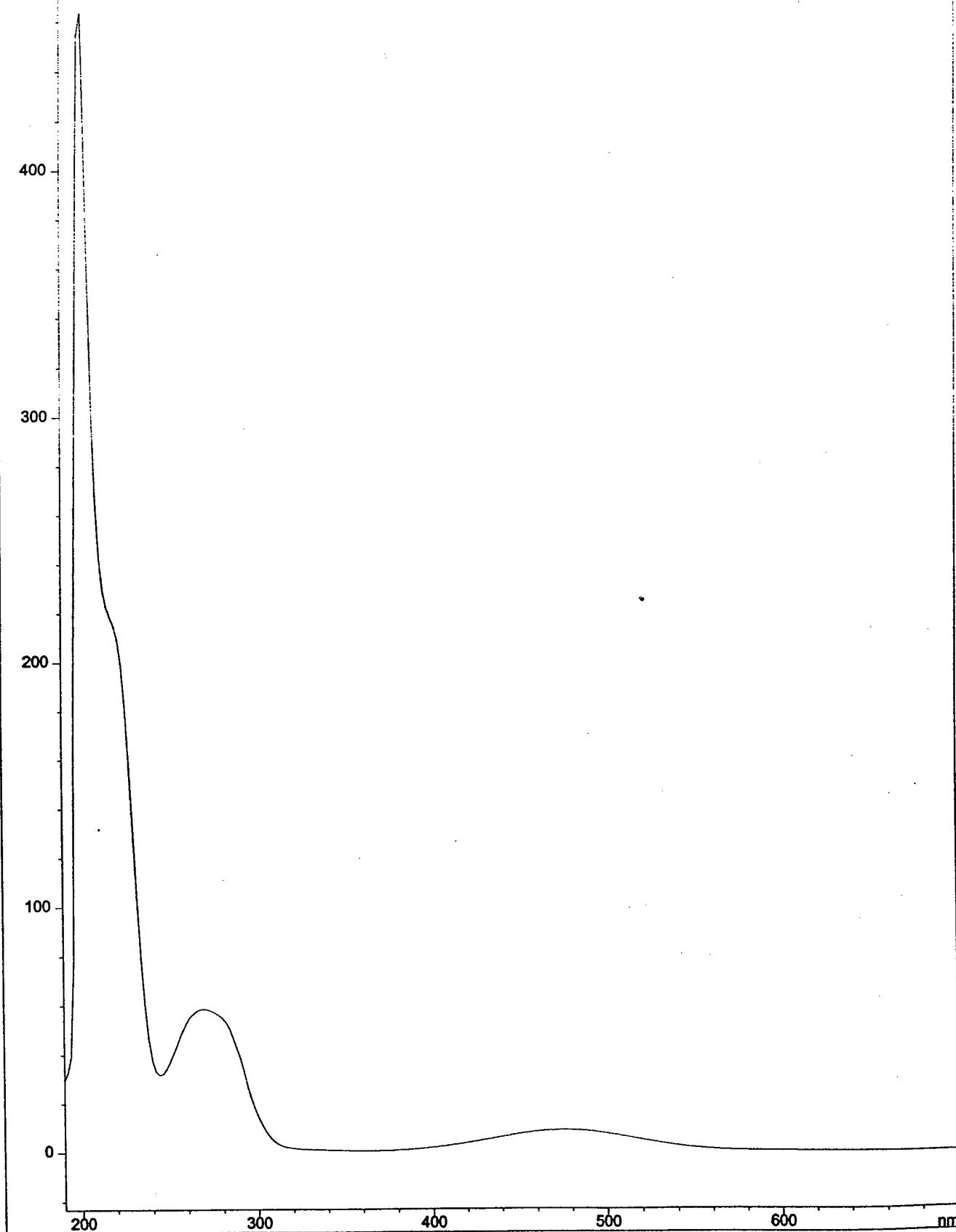
rint of window 39: UV Apex spectrum of Peak 17.713 of EGFSG007.D

UV Apex spectrum of Peak 17.713 of EGFSG007.D

\*DAD1, 17.714 (454 mAU, -) Ref=16.840 & 18.514 of EGFSG007.D

mAU

Figure 2C



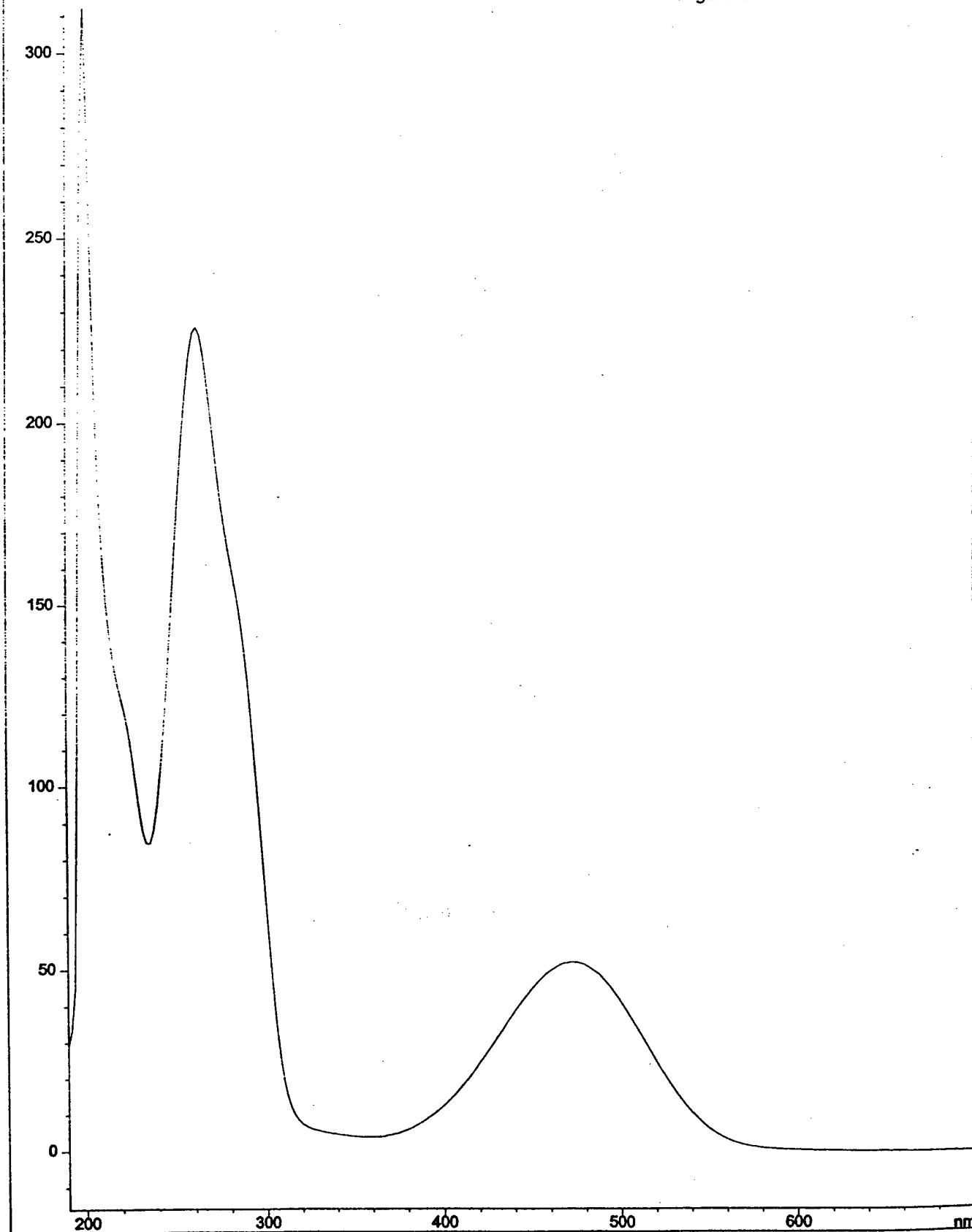
:int of window 39: UV Apex spectrum of Peak 22.442 of EGFSG007.D

UV Apex spectrum of Peak 22.442 of EGFSG007.D

\*DAD1, 22.440(313 mAU, -) Ref=12.874 & 51.480 of EGFSG007.D

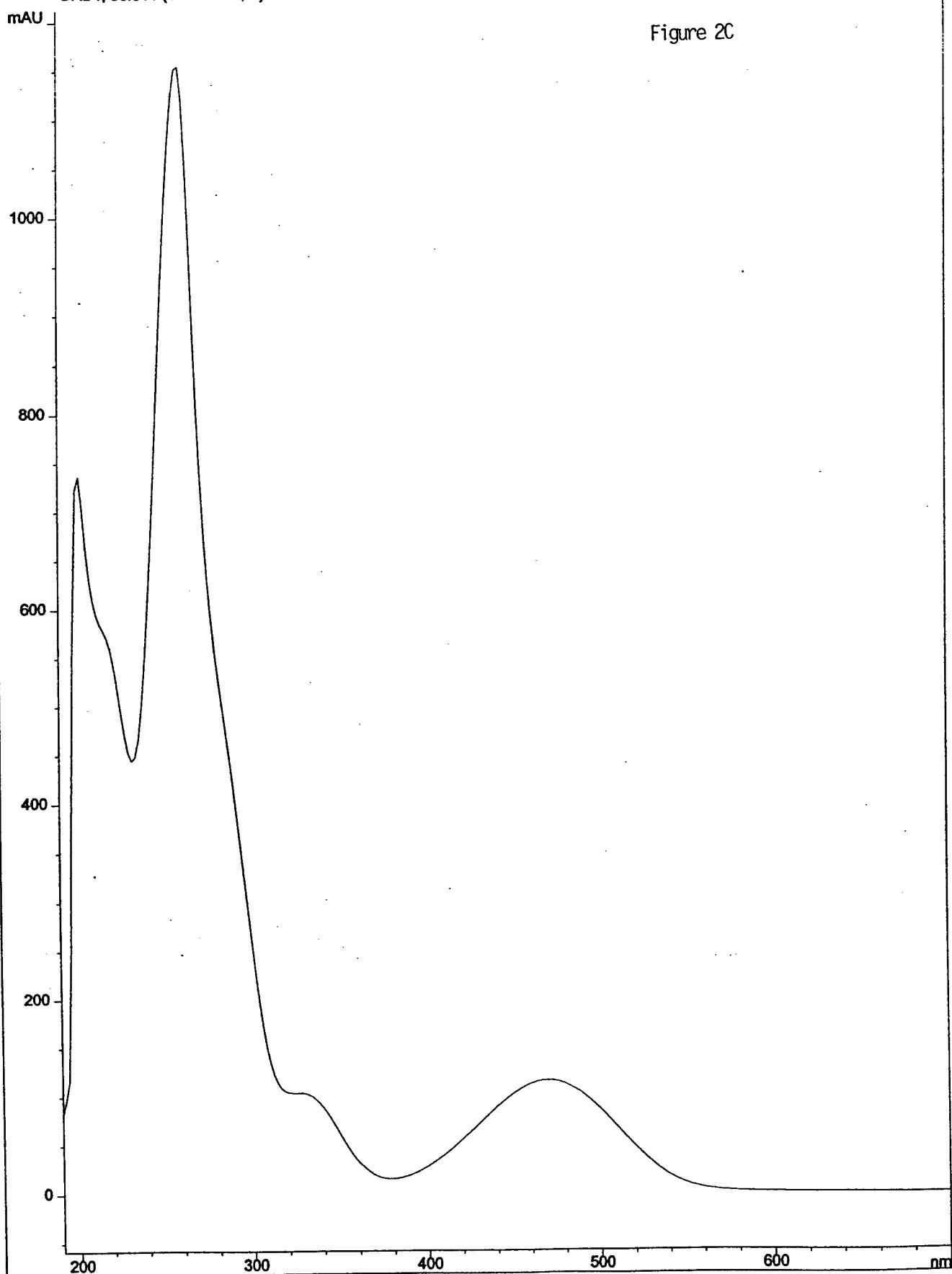
mAU

Figure 2C



UV Apex spectrum of Peak 36.814 of EGFSG007.D

\*DAD1, 36.814 (1155 mAU, -) Ref=12.874 & 51.480 of EGFSG007.D



EGF/SAN-Gen 1:4, 1:10. 16.5 hr LWUV. 5 mg/mL origina

1.  
5/18/99. Analytical III.

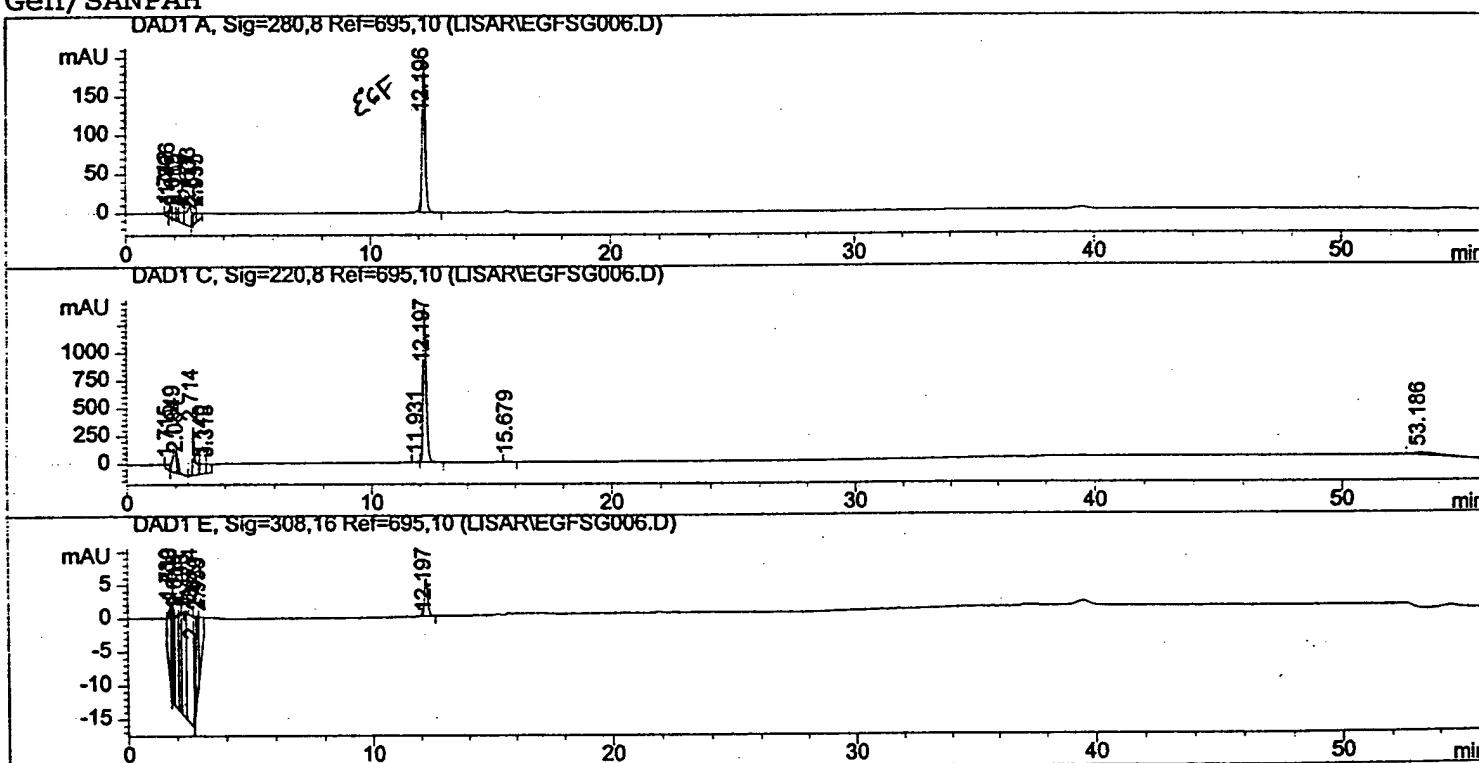
A: H<sub>2</sub>O, 0.1%TFA, 0.1%TEA. D: 80% ACN, 20% H<sub>2</sub>O, 0.1% TFA.

Gradient elution: t=0, 20% D; t=5, 30% D; t=9, 38% D;  
t=20, 43.5% D; t=35, 100% D; t=50, 100% D; t=55, 20% D;  
t=56, stop. Flow = 1 mL/min.

=====

Injection Date	: 5/19/99 8:49:38 AM	Seq. Line	: 13
Sample Name	: EGF/SAN-Gen III	Vial	: 33
Acq. Operator	: Lisa Kuehn	Inj	: 1
		Inj Volume	: 50 $\mu$ L

Acq. Method	: C:\HPCHEM\1\METHODS\LISAEGF.M
Last changed	: 5/12/99 9:27:49 AM by Lisa Kuehn
Analysis Method	: C:\HPCHEM\1\METHODS\LISAGEN3.M
Last changed	: 5/11/99 2:46:56 PM by L. Kuehn
Gen/SANPAH	



**Figure 2D** - Figure 2D is a reverse-phase HPLC trace of fraction III shown in Figure 1. The peak with a retention time of 12.196 min. and a UV spectrum characteristic of unmodified EGF verifies that the size-exclusion chromatography is able to remove a significant amount of the free EGF remaining in the conjugation mixture.

UV Apex spectrum of Peak 12.196 of EGFSG006.D

\*DAD1, 12.194 (1520 mAU, -) Ref=11.334 & 12.620 of EGFSG006.D

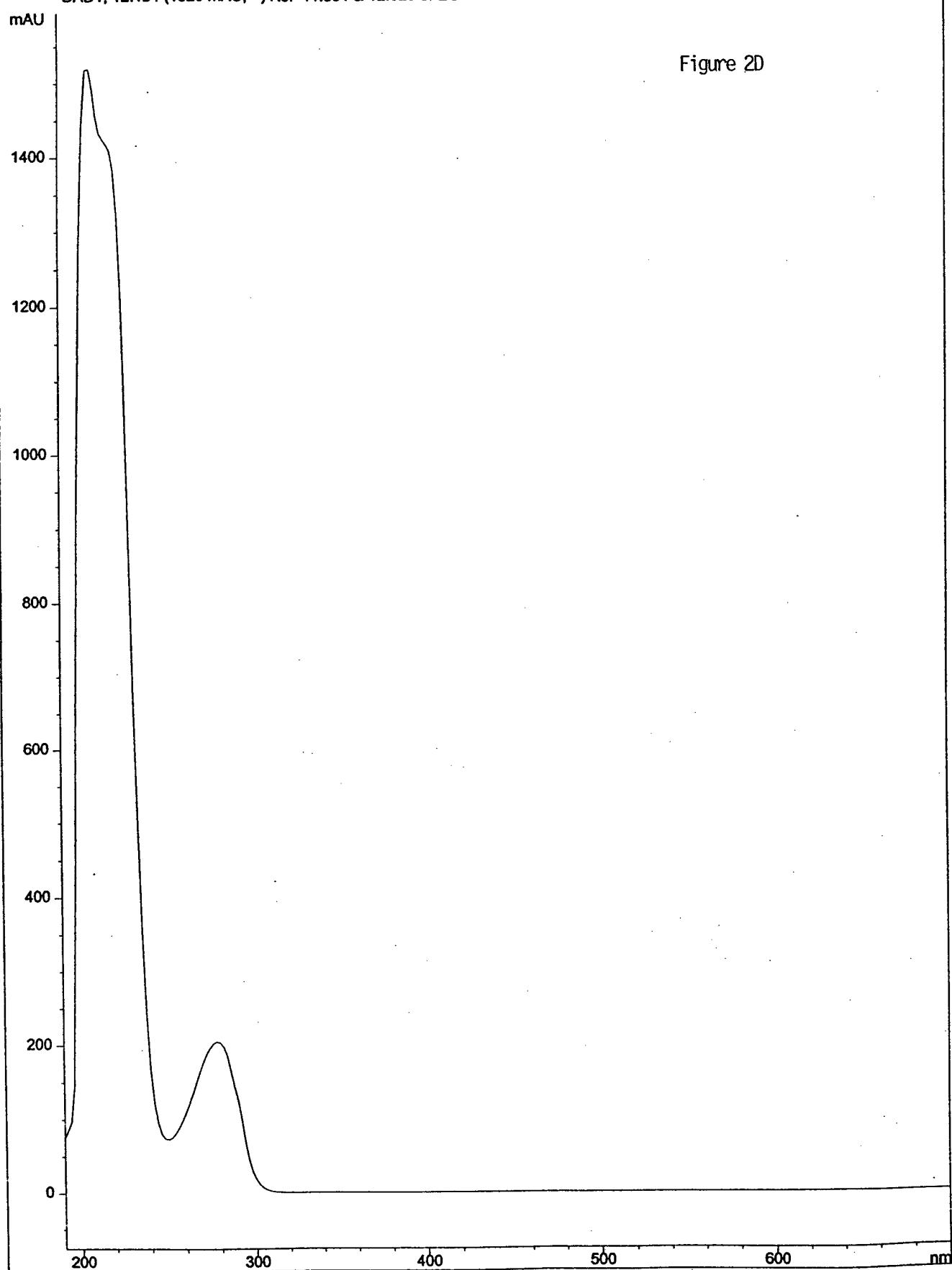
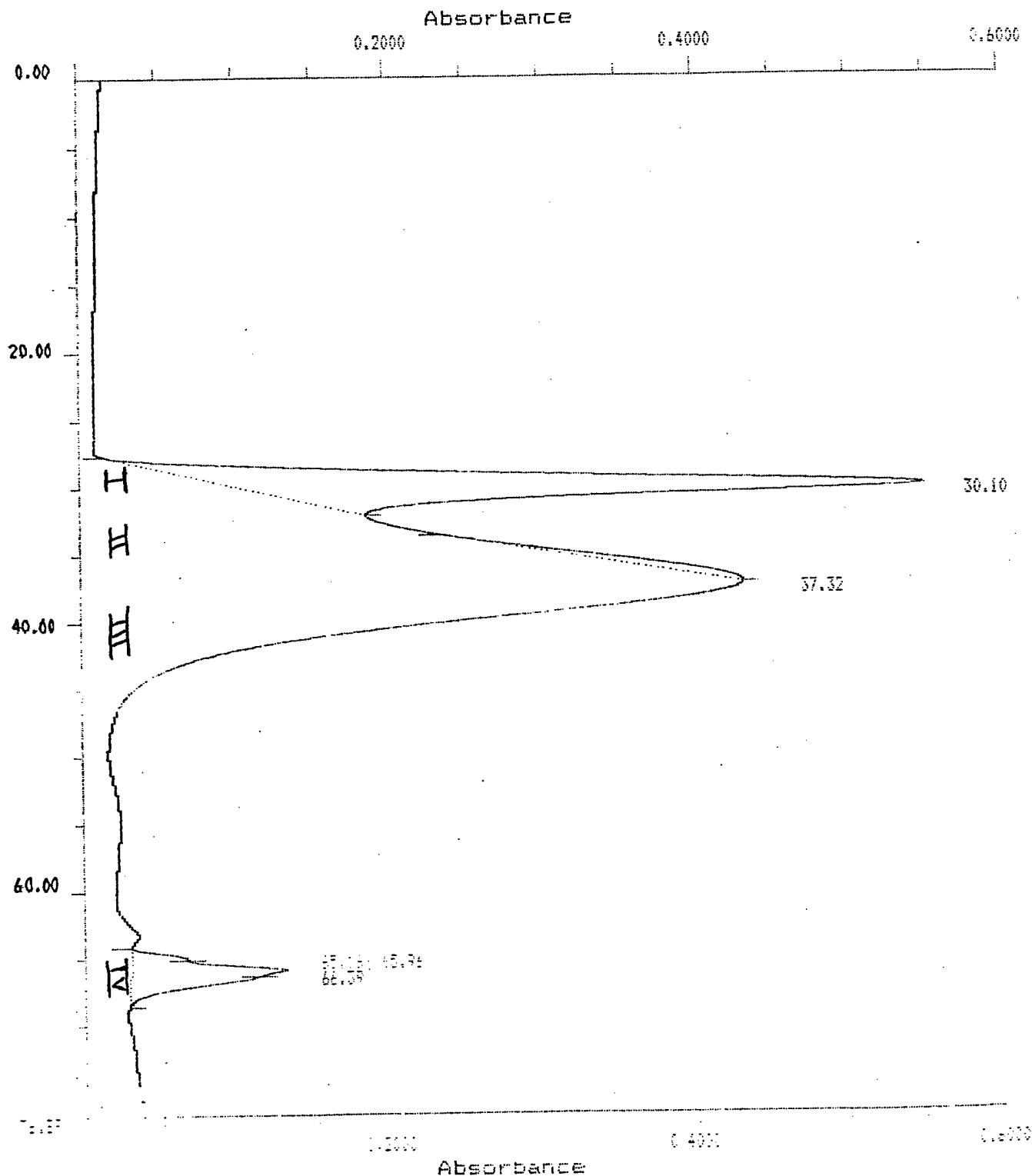
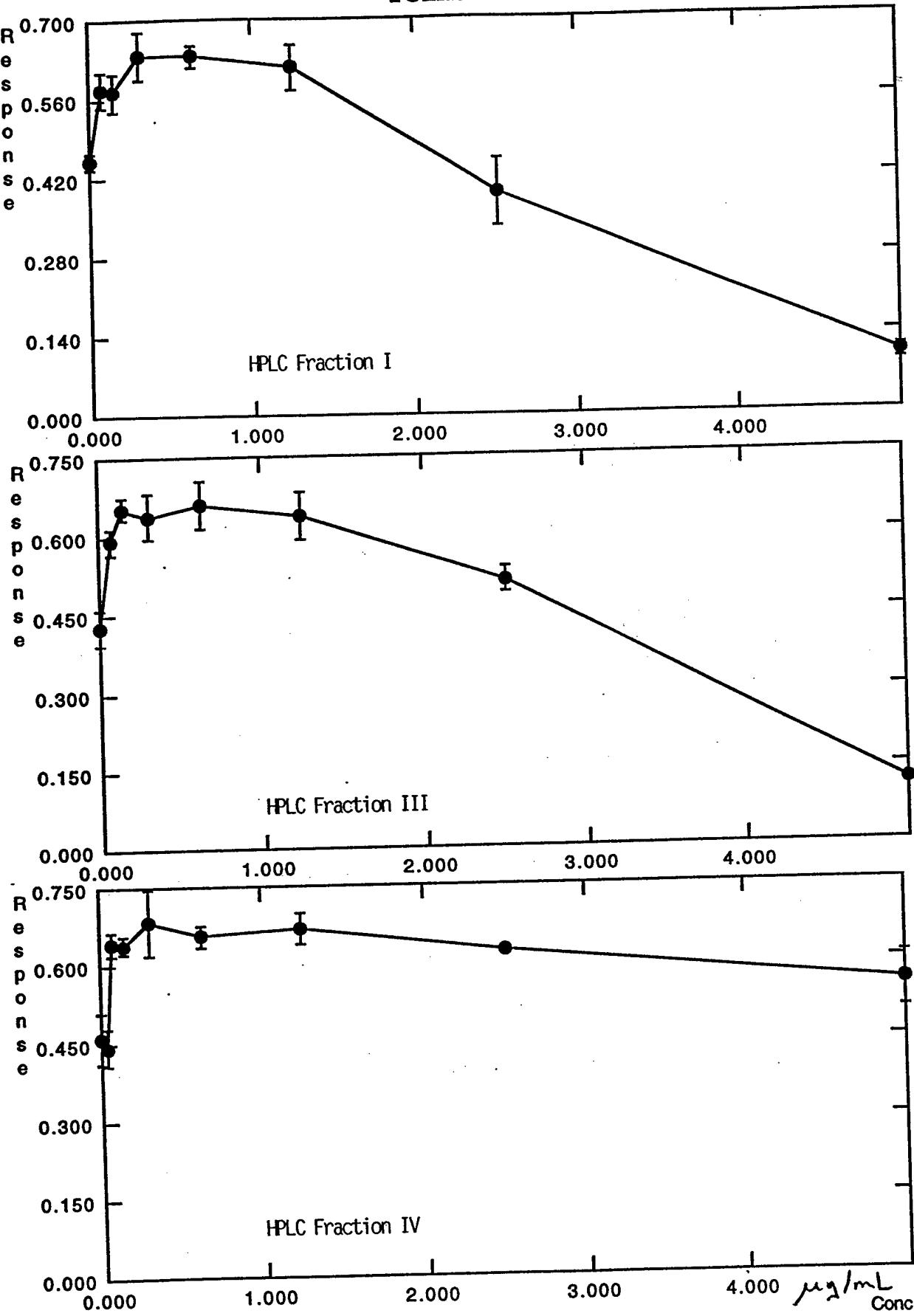


Figure 2D

**Figure 3A** shows a preparative size-exclusion purification of an EGF-Genistein conjugate prepared using the prephotolyzed SANPAH/Genistein mixture. Fractions were isolated and tested against the BT-20 breast cancer cell line using the MTT assay(**Figure 3B** ). In the MTT assay, the greater the response(y-axis), the greater the cell viability.



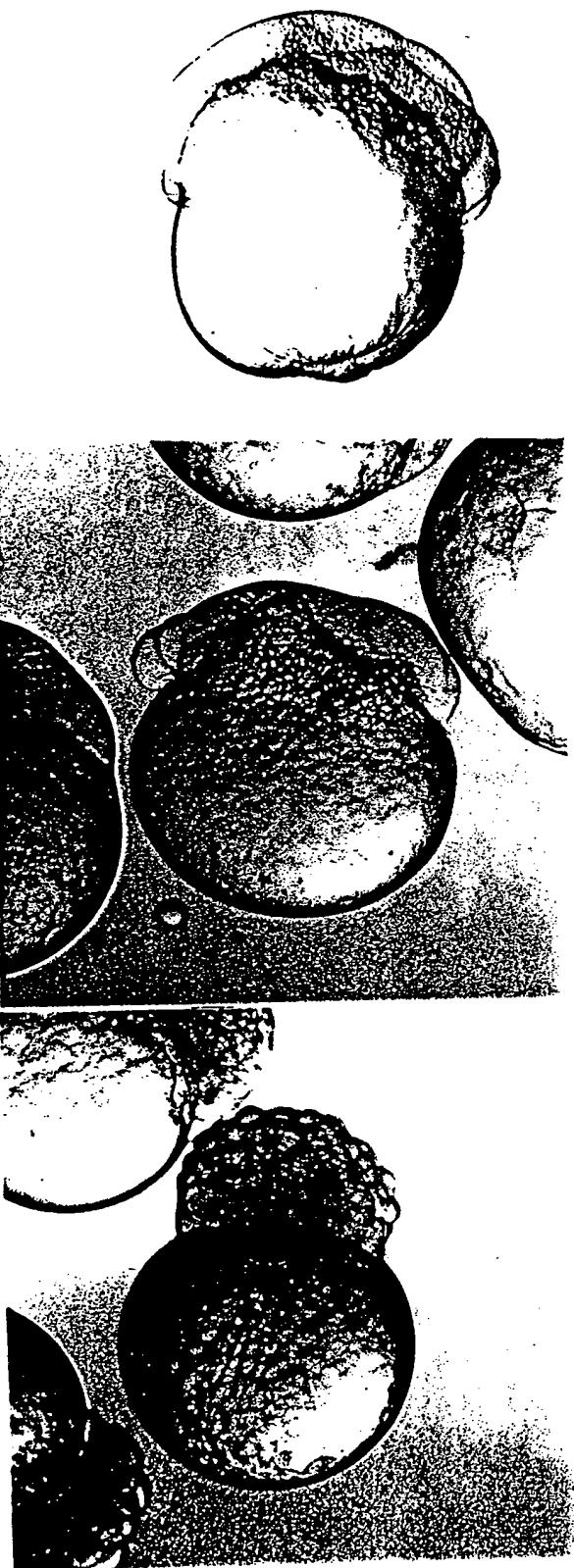
### Point to Point



Point to Point

Figure 3B - MTT Assay of EGF-Genistein HPLC Fractions Incubated with BT-20 Cells

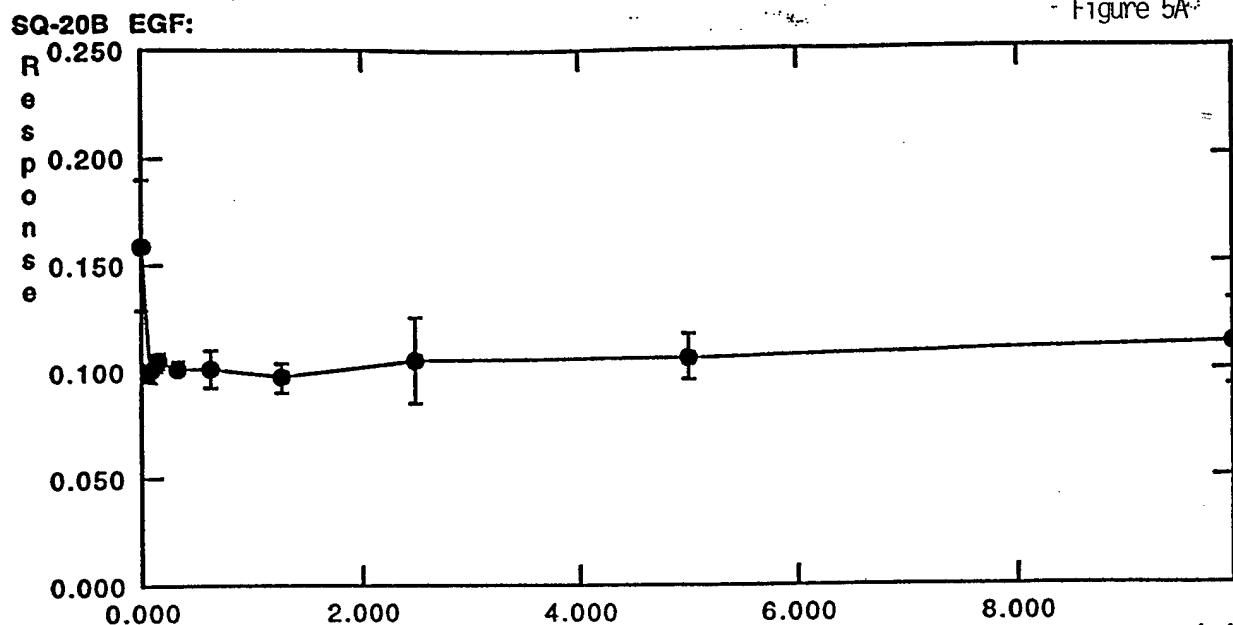
**Figure 4A** shows a zebra fish embryo treated with 50 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 5:1 ratio of Genistein to ANB-NOS. Cell lysis is evident after one hr of incubation.



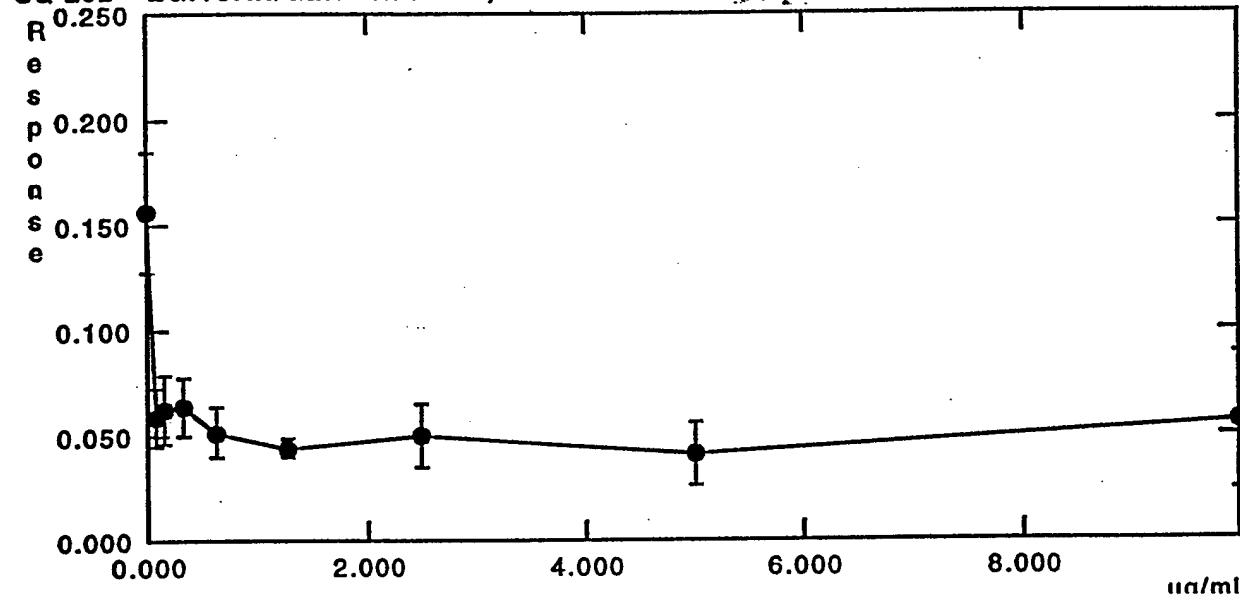
**Figure 4B** shows zebra fish embryos treated with 25 ug/mL of EGF-Gen prepared using a 6.25 hr prephotolysis mixture containing a 10:1 ratio of Genistein to ANB-NOS. Cell lysis is present here as well.

**Figure 4C** Zebra fish embryo showing normal development.

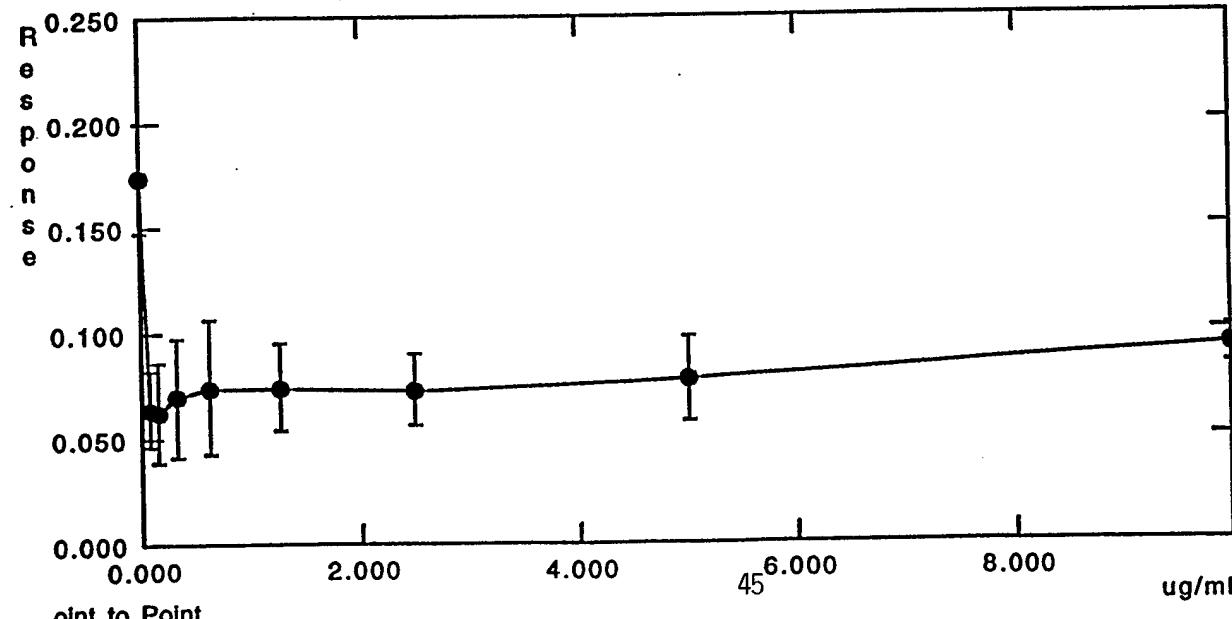
Figure 5A



SQ-20B EGF/SAN/GEN NPP1-10,1-20:



SQ-20B EGF/SAN/P97 1-10,1-5 NPP:



oint to Point

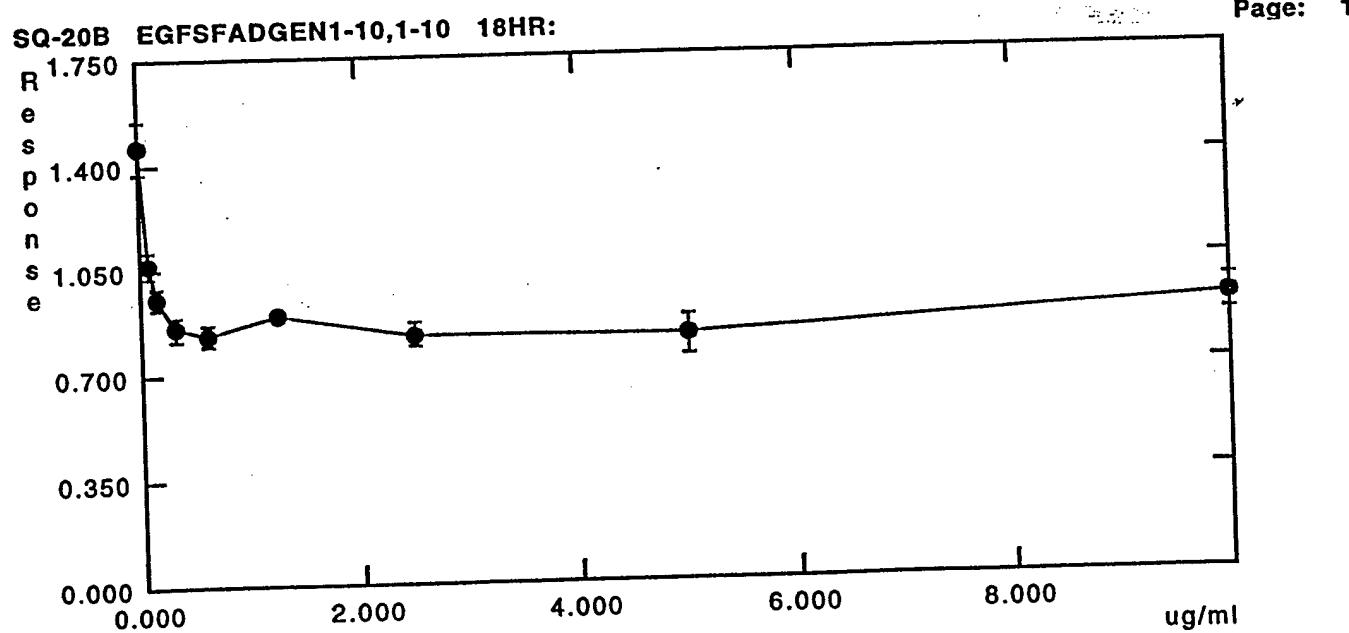
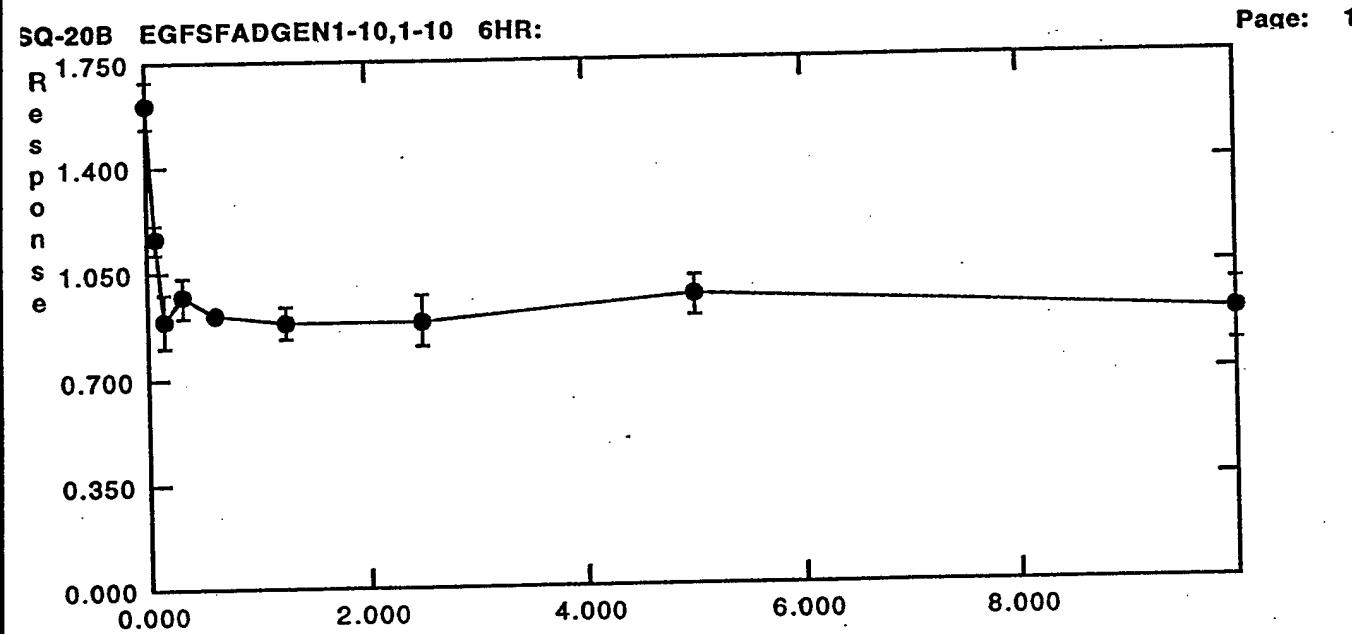
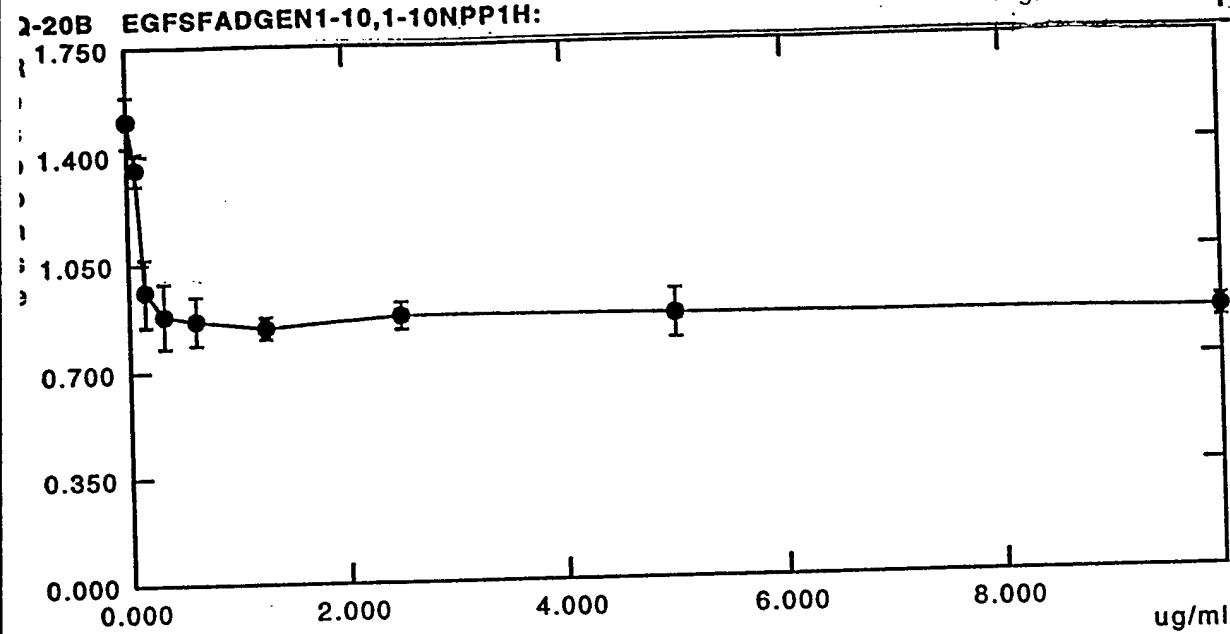
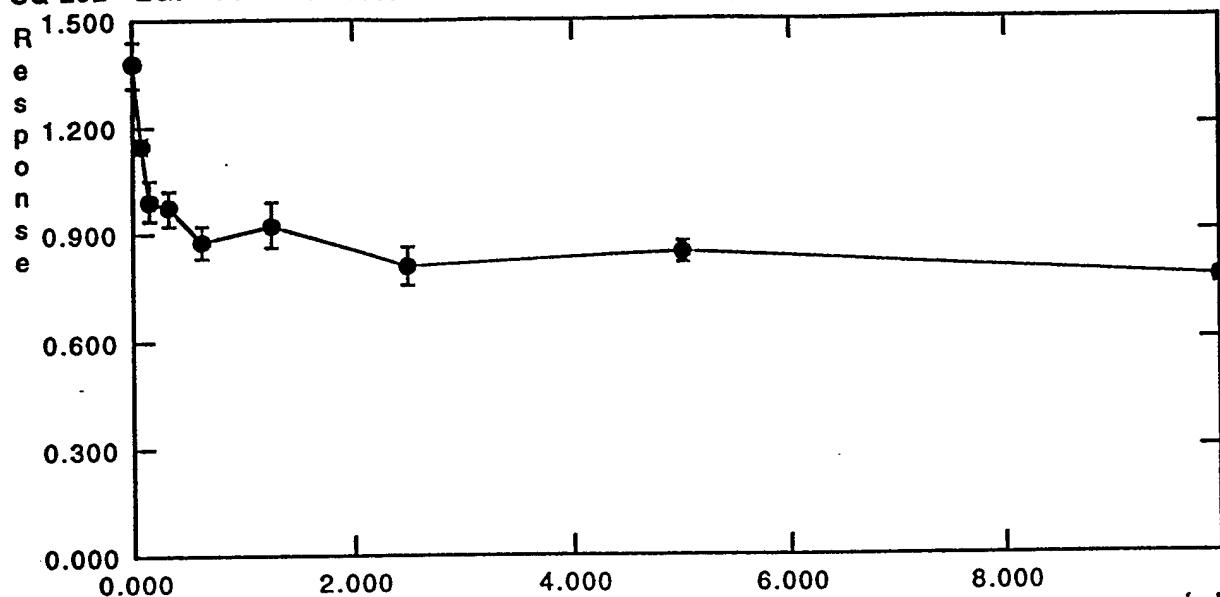


Figure 5C

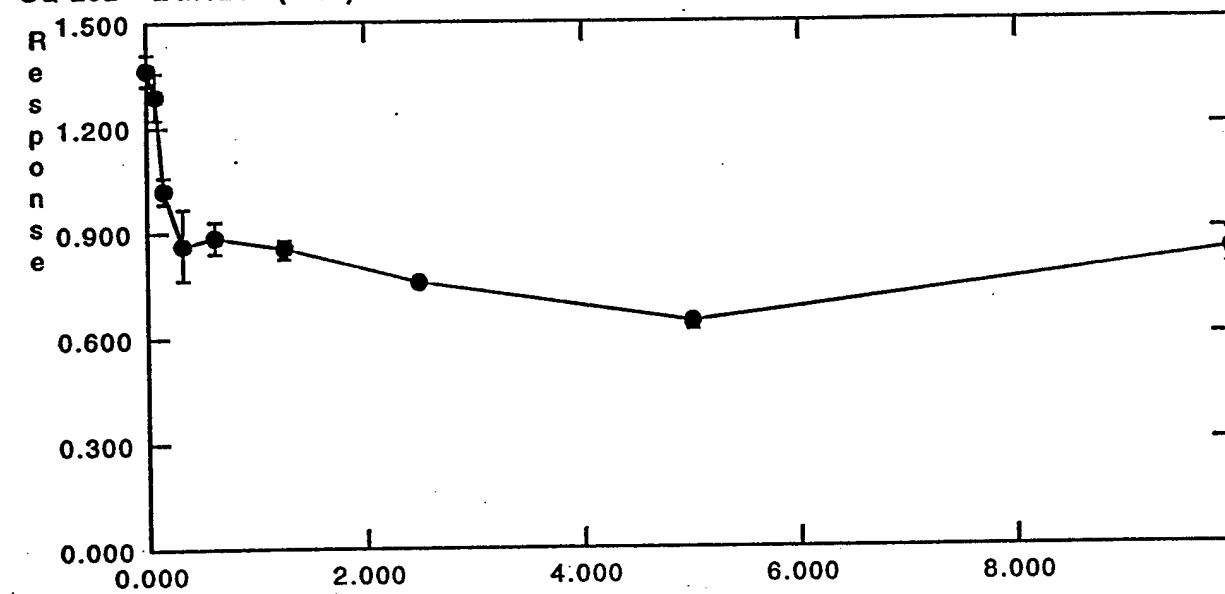
Page: 1

SQ-20B EGF PBS 7/27/99:



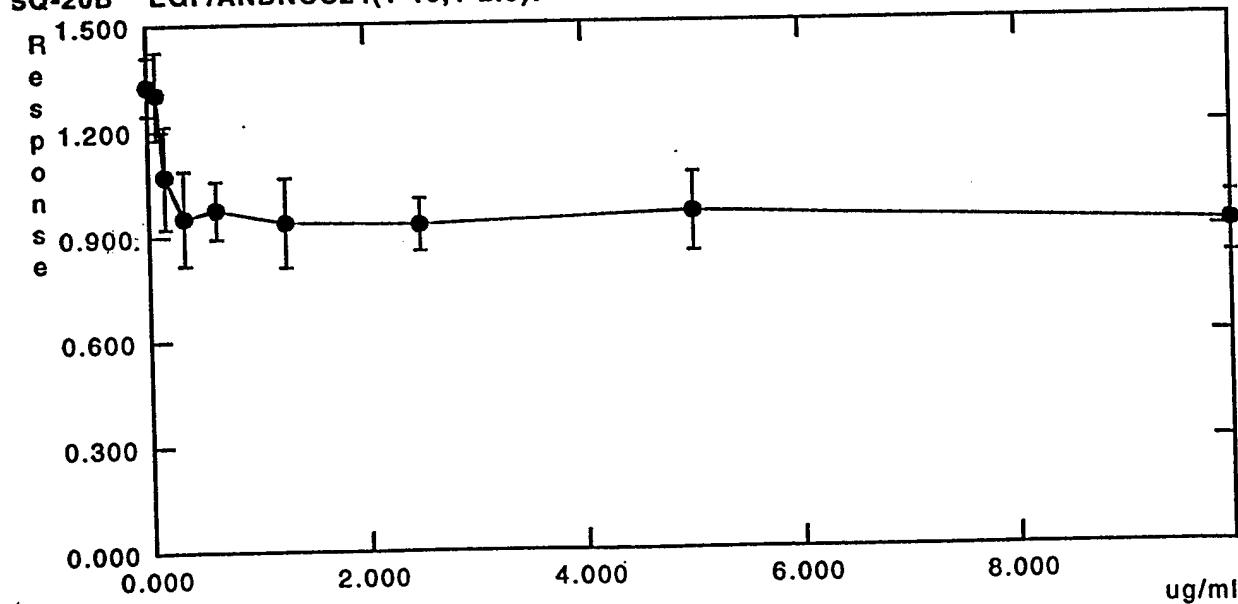
SQ-20B EGF/24 (1-10) 7-26-99:

Page: 1



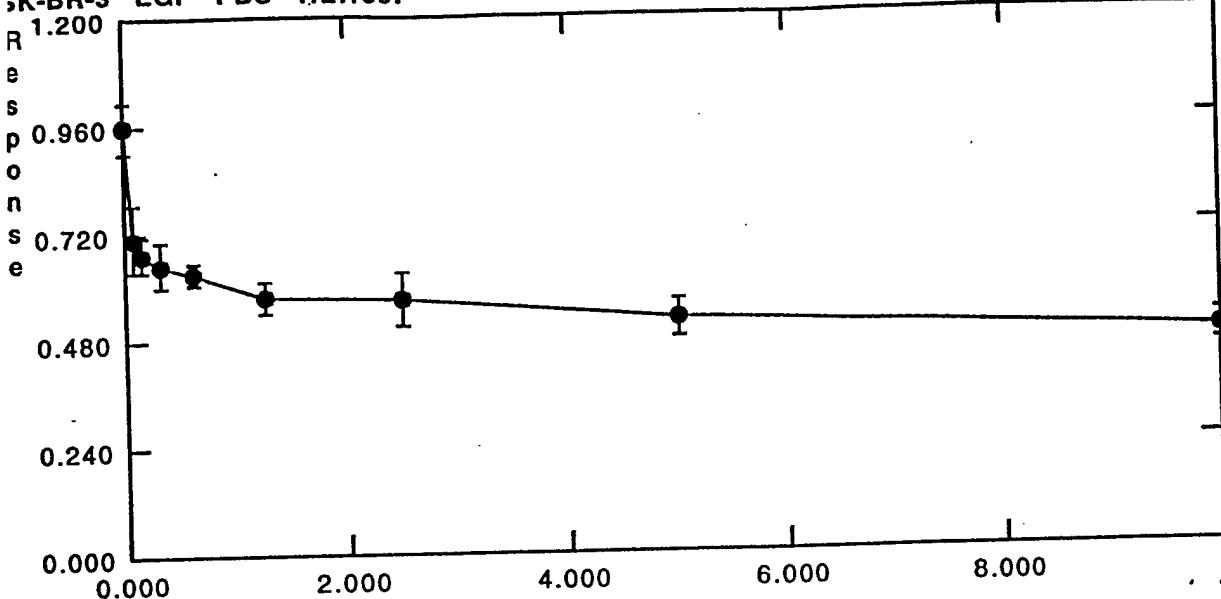
SQ-20B EGF/ANBNOS24(1-10,1-2.5):

Page: 1

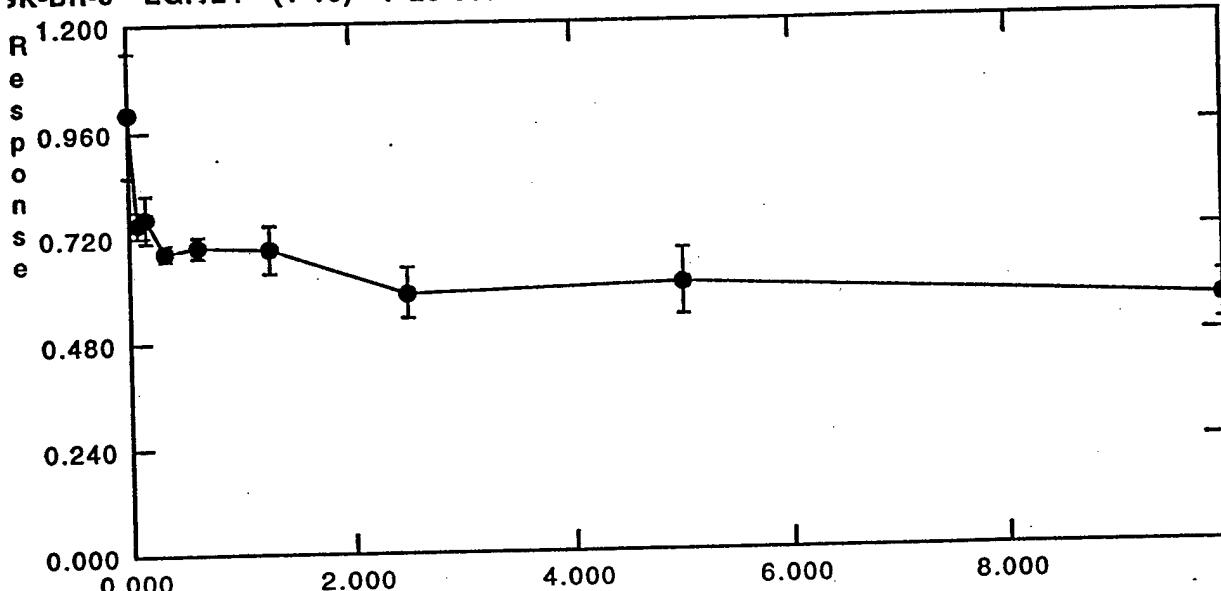


Point to Point

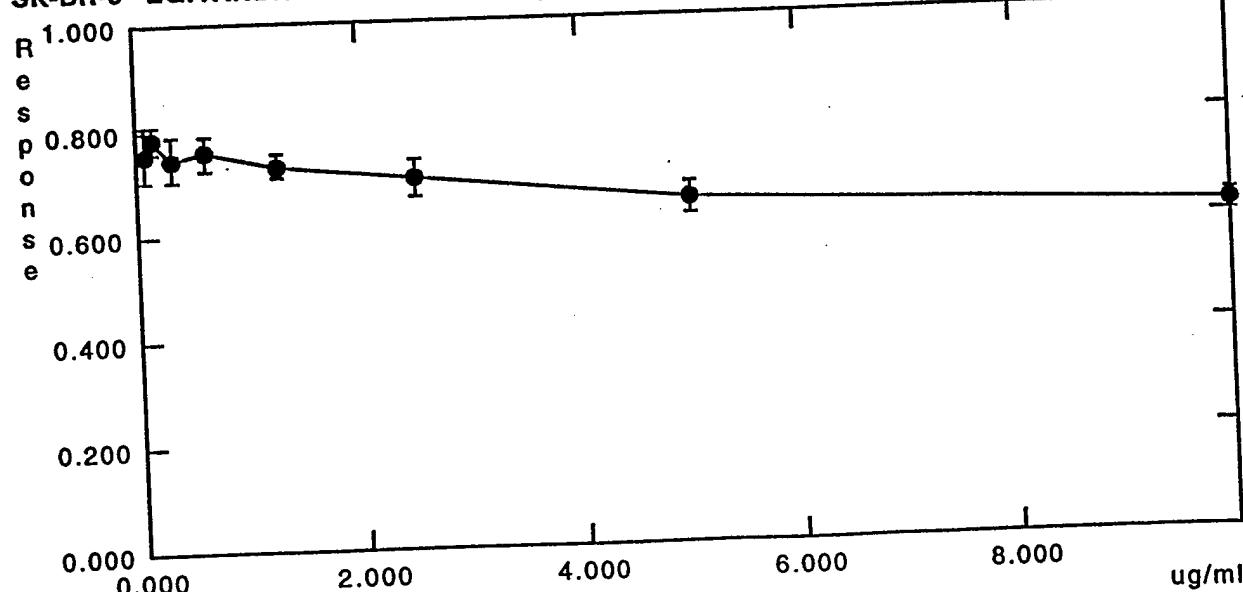
SK-BR-3 EGF PBS 7/27/99:



SK-BR-3 EGF/24 (1-10) 7-26-99:



SK-BR-3 EGF/ANBNOS-24 6HRLW:



ug/ml

Point to Point

## Appendix II

**Monkey 68-K**

**Animal Identification and Necropsy Number:** 68-K

**GROSS OBSERVATIONS:**

<b>Integumentary:</b>	No gross lesions (NGL)
<b>Cardiovascular:</b>	There are hemorrhages in the subcutis adjacent to venipuncture sites.
<b>Respiratory:</b>	NGL
<b>Alimentary:</b>	NGL
<b>Urinary:</b>	NGL
<b>Genital:</b>	NGL
<b>Hemolymphopoietic:</b>	NGL
<b>Endocrine:</b>	NGL
<b>Musculoskeletal:</b>	There are injection sites in the skeletal muscle of the thighs.
<b>Nervous and special senses</b>	NGL

**HISTOPATH OBSERVATIONS:** Pending

**Animal Identification:**  
**Species:**

**68-K**  
***M. fasciculata***

**Tissues submitted for Histopathology:**

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
- 31.

**ANIMAL NECROPSY REPORT**  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

**HISTOPATHOLOGY REPORT**

DATE OF REPORT	9/17/99	AGE	adult
DATE OF NECROPSY	9/30/98	SEX	f
NECROPSY NUMBER	68-K	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-K	NO. of ANIMALS	1

**HISTORY:** Test-article toxicity study

**KEYWORDS:** Genistein, epidermal growth factor

**FINAL DIAGNOSES:** No evidence of test article-related deleterious effects

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** There are no significant lesions.

Pathologist:



Roland Gunther

Roland Gunther, DVM-PhD, ACVP

**HISTOPATH OBSERVATIONS::**

**Integumentary:**

There are no significant lesions (NSL) in multiple sections of skin, lip and eyelid.

**Cardiovascular:**

NSL- heart, aorta

**Respiratory:**

NSL- lung, trachea

**Alimentary:**

NSL- tongue, esophagus, stomach, large and small intestine, liver, pancreas

**Urinary:**

NSL- kidneys, urinary bladder

**Genital:**

NSL- ovary, uterus

**Hemolymphopoietic:**

NSL- spleen, bone marrow, lymph nodes

**Endocrine:**

NSL- pancreas, thyroid, adrenal

**Musculoskeletal:**

NSL- skeletal muscle, bone

**Nervous and special senses**

NSL- multiple areas of brain and spinal cord, peripheral nerve, eye (lens not examined)

**Monkey 68-I**

**ANIMAL NECROPSY REPORT**  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

**GROSS REPORT**

DATE OF REPORT	10/1/98	AGE	adult
DATE OF NECROPSY	9/30/98	SEX	f
NECROPSY NUMBER	68-I	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-I	NO. of ANIMALS	1

**HISTORY:** test-article toxicity study

**KEYWORDS:** genistein, epidermal growth factor

**FINAL DIAGNOSES:** pending histopathologic evaluation

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** There are no significant gross lesions.

Pathologist:



Roland Gunther, DVM-PhD, ACVP

Animal Identification and Necropsy Number: 68-I

**GROSS OBSERVATIONS:**

<b>Integumentary:</b>	No gross lesions (NGL)
<b>Cardiovascular:</b>	There are hemorrhages in the subcutis adjacent to venipuncture sites.
<b>Respiratory:</b>	NGL
<b>Alimentary:</b>	NGL
<b>Urinary:</b>	NGL
<b>Genital:</b>	NGL
<b>Hemolymphopoietic:</b>	NGL
<b>Endocrine:</b>	NGL
<b>Musculoskeletal:</b>	There are injection sites in the skeletal muscle of the thighs.
<b>Nervous and special senses</b>	NGL

**Animal Identification:** 68-I  
**Species:** *M. fasciculata*

**Tissues submitted for Histopathology:**

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
- 31.

**ANIMAL NECROPSY REPORT**  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

**HISTOPATHOLOGY REPORT**

<b>DATE OF REPORT</b>	9/17/99	<b>AGE</b>	adult
<b>DATE OF NECROPSY</b>	9/30/98	<b>SEX</b>	f
<b>NECROPSY NUMBER</b>	68-I	<b>SUPPLIER</b>	
<b>INVESTIGATOR</b>	Gunther	<b>PATHOLOGIST</b>	R. Gunther
<b>DEPARTMENT</b>	RAR	<b>PM INTERVAL</b>	>30 min
<b>SPECIES</b>	M. fasciculata	<b>LAB NUMBER</b>	
<b>BREED/STRAIN</b>		<b>LAB TESTS</b>	
<b>ANIMAL ID</b>	68-I	<b>NO. of ANIMALS</b>	1

**HISTORY:** test-article toxicity study

**KEYWORDS:** genistein, epidermal growth factor

**FINAL DIAGNOSES:** No evidence of test article-related deleterious effects

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** No significant lesions are found in any tissue.

Pathologist:



Roland Gunther, DVM-PhD, ACVP

**HISTOPATH OBSERVATIONS:**

**Integumentary:** There are no significant lesions (NSL) in multiple sections of skin, eyelid and lip.

**Cardiovascular:** NSL-heart, aorta

**Respiratory:** NSL- lung and trachea

**Alimentary:** NSL- tongue, esophagus, stomach, large and small intestine, liver, pancreas

**Urinary:** NSL- kidneys, urinary bladder

**Genital:** NSL- ovary, uterus

**Hemolymphopoietic:** NSL- spleen, lymph nodes, bone marrow

**Endocrine:** NSL- pancreas, adrenal, thyroid, parathyroid

**Musculoskeletal:** NSL- skeletal muscle, rib

**Nervous and special senses** NSL- multiple areas of brain and spinal cord, peripheral nerve, eye (lens not examined)

**Monkey 68-N**

**ANIMAL NECROPSY REPORT**  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

**GROSS REPORT**

DATE OF REPORT	10/13/98	AGE	adult
DATE OF NECROPSY	10/13/98	SEX	f
NECROPSY NUMBER	68-N	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-N	NO. of ANIMALS	1

**HISTORY:** test-article toxicity study

**KEYWORDS:** genistein, epidermal growth factor

**FINAL DIAGNOSES:** pending histopathologic evaluation

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** There are no significant gross lesions.

Pathologist:



Roland Gunther, DVM-PhD, ACVP

**Animal Identification and Necropsy Number:** 68-N

**GROSS OBSERVATIONS:**

<b>Integumentary:</b>	No gross lesions (NGL)
<b>Cardiovascular:</b>	There are hemorrhages in the subcutis adjacent to venipuncture sites.
<b>Respiratory:</b>	NGL
<b>Alimentary:</b>	NGL
<b>Urinary:</b>	NGL
<b>Genital:</b>	NGL
<b>Hemolymphopoietic:</b>	NGL
<b>Endocrine:</b>	NGL
<b>Musculoskeletal:</b>	There are injection sites in the skeletal muscle of the thighs.
<b>Nervous and special senses</b>	NGL

**HISTOPATH OBSERVATIONS:** Pending

**Animal Identification:** 68-N  
**Species:** *M. fasciculata*

**Tissues submitted for Histopathology:**

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
- 31.

**ANIMAL NECROPSY REPORT**  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

**HISTOPATHOLOGY REPORT**

DATE OF REPORT	9/17/99	AGE	adult
DATE OF NECROPSY	10/13/98	SEX	f
NECROPSY NUMBER	68-N	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-N	NO. of ANIMALS	1

**HISTORY:** Test-article toxicity study

**KEYWORDS:** Genistein, epidermal growth factor

**FINAL DIAGNOSES:** No evidence of test article-related deleterious effects

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** There are no significant lesions.

**Pathologist:**



Roland Gunther, DVM-PhD, ACVP

**Animal Identification and Necropsy Number:** 68-N

**HISTOPATH OBSERVATIONS:**

**Integumentary:** There are no significant lesions (NSL) in multiple sections of skin, lip and eyelid.  
**Cardiovascular:** NSL- heart, aorta  
**Respiratory:** NSL-lung, trachea  
**Alimentary:** NSL- tongue, esophagus, stomach, large and small intestine, pancreas, liver  
**Urinary:** NSL- There is a mild increase in glomerular mesangial matrix. No lesions in the urinary bladder.  
**Genital:** NSL- ovary, uterus  
**Hemolymphopoietic:** NSL-lymph nodes, spleen, bone marrow  
**Endocrine:** NSL-thyroid, parathyroid, pancreas, adrenal  
**Musculoskeletal:** NSL- skeletal muscle, bone  
**Nervous and special senses** NSL- multiple areas of brain and spinal cord, peripheral nerve, eye

**Monkey 68-J**

**ANIMAL NECROPSY REPORT**  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

**GROSS REPORT**

DATE OF REPORT	10/13/98	AGE	adult
DATE OF NECROPSY	10/13/98	SEX	f
NECROPSY NUMBER	68-J	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-J	NO. of ANIMALS	1

**HISTORY:** test-article toxicity study

**KEYWORDS:** genistein, epidermal growth factor

**FINAL DIAGNOSES:** pending histopathologic evaluation

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** There are no significant gross lesions.

Pathologist:



Roland Gunther, DVM-PhD, ACVP

**Animal Identification and Necropsy Number:** 68-J

**GROSS OBSERVATIONS:**

<b>Integumentary:</b>	No gross lesions (NGL)
<b>Cardiovascular:</b>	There are hemorrhages in the subcutis adjacent to venipuncture sites.
<b>Respiratory:</b>	NGL
<b>Alimentary:</b>	NGL
<b>Urinary:</b>	NGL
<b>Genital:</b>	NGL
<b>Hemolymphopoietic:</b>	NGL
<b>Endocrine:</b>	NGL
<b>Musculoskeletal:</b>	There are injection sites in the skeletal muscle of the thighs.
<b>Nervous and special senses</b>	NGL

**HISTOPATH OBSERVATIONS:** Pending

**Animal Identification:** 68-J  
**Species:** M. fasciculata

**Tissues submitted for Histopathology:**

1. adrenal glands
2. aorta
3. bone (decalcified)
4. bone marrow
5. brain-cerebellum & medulla
6. brain - forebrain
7. brain - midbrain region
8. eye
9. eyelid
10. heart (LV, RV, IVS)
11. kidney
12. large intestine
13. liver
14. lung
15. lymph nodes
16. ovary
17. pancreas
18. peripheral nerve (sciatic nerve and brachial plexus)
19. pituitary gland
20. salivary glands
21. skeletal muscle
22. skin
23. small intestine
24. spinal cord-cervical, thoracic lumbar, cauda equinae
25. spleen
26. stomach
27. thyroid glands
28. tongue
29. urinary bladder
30. uterus
- 31.

**HISTOLOGIC OBSERVATIONS:**

**Integumentary:** There are no significant (NSL) lesions in multiple sections of skin, eyelid and lip.  
**Cardiovascular:** NSL- heart and aorta  
**Respiratory:** NSL- lung, trachea  
**Alimentary:** NSL- esophagus, tongue, stomach, large and small intestine, pancreas, liver  
**Urinary:** NSL- kidneys, urinary bladder  
**Genital:** NSL- ovary, uterus  
**Hemolymphopoietic:** NSL- spleen, lymph nodes, bone marrow  
**Endocrine:** NSL- pancreas, adrenal, thyroid  
**Musculoskeletal:** NSL- skeletal muscle, bone  
**Nervous and special senses** NSL- multiple areas of brain and spinal cord, peripheral nerve, eye (lens not examined)

ANIMAL NECROPSY REPORT  
RESEARCH ANIMAL RESOURCES  
UNIT OF COMPARATIVE MEDICINE  
ACADEMIC HEALTH SCIENCES  
UNIVERSITY OF MINNESOTA

HISTOPATHOLOGIC REPORT

DATE OF REPORT	9/17/99	AGE	adult
DATE OF NECROPSY	10/13/98	SEX	f
NECROPSY NUMBER	68-J	SUPPLIER	
INVESTIGATOR	Gunther	PATHOLOGIST	R. Gunther
DEPARTMENT	RAR	PM INTERVAL	>30 min
SPECIES	M. fasciculata	LAB NUMBER	
BREED/STRAIN		LAB TESTS	
ANIMAL ID	68-J	NO. of ANIMALS	1

**HISTORY:** test-article toxicity study

**KEYWORDS:** genistein, epidermal growth factor

**FINAL DIAGNOSES:** No evidence of test article-related deleterious effects

**CAUSE OF DEATH:** Euthanasia

**COMMENTS:** There are no significant lesions.

Pathologist:



Roland Gunther, DVM-PhD, ACVP

### **Appendix III**



## IN VIVO TOXICITY OF CHEMOTHERAPY DRUGS +/- EGF-GEN (4/19/99)

### MATERIALS AND METHODS

**Toxicity Studies in SCID Mice.** All SCID mice used in this toxicity study were obtained from the specific pathogen free (SPF) breeding facilities of Taconic at 4 weeks of age. The mice were housed in the animal housing facility of the Hughes Institute. All husbandry and experimental contact made with the mice maintained SPF conditions. The mice were kept in microisolator cages (Lab Products, Inc., Maywood, NY) containing autoclaved food, water and bedding.

In this toxicity study, 14 weighed five week old female SCID mice averaging 17.7 g were administered intraperitoneal bolus injections of one of four chemotherapy drugs in 0.2 mL sterile water solution. Groups of 4 mice received treatments of one of the following: 17 mg/kg/d x 5d Taxol (0.34 mg/ms/d x 5d), 8 mg/kg/d x 1d Adriamycin (0.16 mg/ms/d), or 1 mg/kg/d x 5d Methyltrexate (0.02 mg/ms/d x 5d). Two mice from each of the aforementioned groups received 4 µg/d x 10d EGF-Gen starting the day following the last chemotherapy treatment for each drug. Two additional mice received 50 mg/kg/d x 2d Cytoxan (1 mg/ms/d x 2d). No sedation or anesthesia was used throughout the treatment period. Mice were monitored daily for mortality for determination of day 30 LD<sub>50</sub> values. At time of sacrifice or death, mice will be weighed. Multiple organs were collected within 4 hours after death, grossly examined, and processed for histopathologic examination. Mice surviving 30 days post-treatment were sacrificed and the tissues were immediately collected and preserved in 10% neutral phosphate buffered formalin.

### RESULTS

*There were no immediate adverse affects observed following drug administration. All surviving mice will be electively sacrificed healthy on day 30 (May 19, 1999).*

*All mice receiving Adriamycin, either alone or in combination with EGF-Gen, died between days 5 - 9. Refer to the experiment table for gross observations taken during dissection. One CTX + EGF-Gen mouse is showing some signs of toxicity (#27456) - scruffy coat, slowed movement. It will be closely examined throughout the weekend.*

*(5/14/99) Mice appear healthy. Mouse #27456 appears to have recovered from the earlier signs of toxicity. All remaining survivors will be sacrificed on Wednesday, May 19, 1999 (day 30).*

*(5/19/99) All surviving mice were sacrificed healthy on day 30, May 19, 1999. All mice were found to be unremarkable at time of sacrifice, with the exception of #27452 (Taxol) and #27464 (MTX + EGF-Gen). Gross observations are noted in the attached experiment table.*

*The mean experimental weight change observed in the Adriamycin and Adriamycin + EGF-Gen groups were -32.8% and -26.3%, respectively. For the five treatment groups that did not sustain any deaths during the experiment, the weight change ranged from 12.4% in the CTX + EGF-Gen group to 20.9% in the Taxol + EGF-Gen group.*

**Table 1. Life-Table Analysis of Survival Data and Statistical Analysis of Weight Change Following Chemotherapy +/- EGF-Gen Intraperitoneal Administration - 4/19/99**

Treatment Group	#	Proportion Surviving (%)						Survival p-value*			
		of Mice	Day 15	Day 30	Taxol	Taxol + EGF-Gen	CTX + EGF-Gen	Adriamycin	Adriamycin + EGF-Gen	MTX	MTX + EGF-Gen
Taxol	2	100 ± 0.0	100 ± 0.0		NA	NA	0.1797	0.1797	NA	NA	NA
Taxol + EGF-Gen	2	100 ± 0.0	100 ± 0.0		NA	NA	0.1797	0.1797	NA	NA	NA
CTX + EGF-Gen	2	100 ± 0.0	100 ± 0.0		NA	NA	0.1797	0.1797	NA	NA	NA
Adriamycin	2	0 ± 0.0	0 ± 0.0		0.1797	0.1797	0.1797	0.1797	0.1797	0.1797	0.1797
Adriamycin + EGF-Gen	2	0 ± 0.0	0 ± 0.0		0.1797	0.1797	0.1797	0.1797	0.1797	0.1797	0.1797
MTX	2	100 ± 0.0	100 ± 0.0		NA	NA	NA	NA	NA	NA	NA
MTX + EGF-Gen	2	100 ± 0.0	100 ± 0.0		NA	NA	0.1797	0.1797	0.1797	0.1797	NA

Treatment Group	#	Mean Weight Change (g) ± SEM						Weight Change p-value*		
		of Mice	Change (g)	Taxol	Taxol + EGF-Gen	CTX + EGF-Gen	Adriamycin	Adriamycin + EGF-Gen	MTX	MTX + EGF-Gen
Taxol	2	3.55 ± 0.35		0.9313	0.4732	0.0024	0.0123	0.7751	0.3441	
Taxol + EGF-Gen	2	3.70 ± 1.50	0.9313		0.5528	0.0250	0.0401	0.8185	0.6266	
CTX + EGF-Gen	2	2.20 ± 1.50	0.4732	0.5528		0.0347	0.0579	0.6045	0.7408	
Adriamycin	2	-5.80 ± 0.30	0.0024	0.0250	0.0347		0.3302	0.0098	0.0046	
Adriamycin + EGF-Gen	2	-4.65 ± 0.85	0.0123	0.0401	0.0579	0.3302		0.0224	0.0171	
MTX	2	3.25 ± 0.85	0.7751	0.8185	0.6045	0.0098	0.0224		0.6929	
MTX + EGF-Gen	2	2.80 ± 0.50	0.3441	0.6266	0.7408	0.0046	0.0171	0.6929		

\*Weight p-value determined by unpaired t-test analysis. A p-value <0.05 was considered significant.

Project Related Study Not Conducted Under This Grant

**Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study.**

**Experiment Date: 4/19/99.**



Date: 8/31/99

Barbara J. Waurzyniak, DVM, MS.

Veterinary Pathologist

Hughes Institute - PreClinical Laboratory

2680 Patton Road

Roseville, MN 55113

Phone: 651-604-9064

Fax: 651-604-9065

**Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.**

**A. MATERIAL AND METHODS:**

**1. The study was performed as follows:**

Beginning on 4/19/99, 5 weeks old female SCID mice received the following treatments.

Group 1: Taxol (TXL): 17 mg/kg/day x 5 days.  
Group 2: Taxol (TXL): 17 mg/kg/day x 5 days + EGF-GEN: 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.  
Group 3: Cytoxan (CTX): 50 mg / kg / day x 2 days + EGF-GEN: 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.  
Group 4: Adriamycin (ADM): 8 mg / kg x 1 day.  
Group 5: Adriamycin (ADM): 8 mg / kg x 1 day + EGF-GEN: 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.  
Group 6: Methyltrexate (MTX): 1 mg / kg / day x 5 days.  
Group 7: Methyltrexate (MTX): 1 mg / kg / day x 5 days + EGF-GEN: 4 µg/day x 10 days, beginning the day following the last chemotherapy treatment.

The group size was 2 mice per group.

No untreated control mice were included in the experiment.

**2. TABLE 1: Mouse Identification and Treatment Table..... 4/19/99**

Group:	1	2	3	4	5	6	7
Treatment	TXL	TXL + EGF-GEN	CTX + EGF-GEN	ADM	ADM + EGF-GEN	MTX	MTX + EGF-GEN
Mouse ID#s:	27451 27452	27543 27454	27455 27456	27457 27458	27459 27460	27461 27462	27463 27464
Total # of mice / group	2	2	2	2	2	2	2
# of mice examined	2	2	2	2	2	2	2

**3. Table 2:..... Outcome (Survival - days):**

Group #	Treatment	Survival (days)
Group 1:	Taxol (TXL)	2/2 (100%) SH at 30 days.
Group 2:	Taxol (TXL)+ EGF-GEN	2/2 (100%) SH at 30 days.
Group 3:	Cytoxan (CTX) + EGF-GEN	2/2 (100%) SH at 30 days.
Group 4:	Adriamycin (ADM)	2/2 (100%) died on day 8 and 9.
Group 5:	Adriamycin (ADM) + EGF-GEN	2/2 (100%) died on day 5 and 7.
Group 6:	Methyltrexate (MTX)	2/2 (100%) SH at 30 days.
Group 7:	Methyltrexate (MTX) + EGF-GEN	2/2 (100%) SH at 30 days.

**3. Clinical Phase, Necropsy and harvesting of tissues:**

- a. The clinical phase, necropsy and harvesting of tissues was performed at the Hughes Institute, 2680 Patton Road, Roseville, MN 55113.
- b. At death, all mice had routine postmortem examinations. Tissues from selected mice were collected, fixed in 10% formalin, and processed for histologic sectioning in a routine manner. The histology slides were stained with Hematoxylin and Eosin.
- c. The histologic evaluation of the tissues and report compilation was done by Barbara J. Waurzyniak, DVM., MS., (veterinary pathologist).

**Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.**

**B. EXPERIMENTAL RESULTS:**

**1. Potential test-agent related lesions (See Table 3):**

- a. Bone Marrow, erythroid, myeloid and megakaryocytic depletion (pancytopenia), marked. Present in 2/2 (100%) of the mice in Group 4 (Adriamycin) and 2/2 (100%) of the mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.
- b. Stomach, focal gastric ulceration and inflammation. Present in 1/2 (50%) of the mice in Group 4 (Adriamycin).
- c. Kidney, acute tubular necrosis, mild, multifocal, renal cortex. Present in 2/2 (100%) of the mice in Group 4 (Adriamycin) and 2/2 (100%) of the mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.
- d. Liver, multifocal hepatic necrosis, mild, acute. Present 1/2 (50%) of the mice in Group 5 (Adriamycin + EGF-GEN). May be caused by Adriamycin or Mouse Hepatitis Virus. Hepatic necrosis was considered unlikely to be caused by EGF-GEN because of the absence of hepatic necrosis in Group 2 (Taxol + EGF-GEN), Group 3 (Cytoxin + EGF-GEN) and Group 7 (Methotrexate + EGF-GEN).
- e. Ovary, follicular atrophy, moderate to marked. Present in 1/1 (100%) of the examined mice in Group 4 (Adriamycin) and 1/1 (100%) of the examined mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.
- f. Spleen, reduced hematopoiesis in the red pulp. Present in 1/1 (100%) of the examined mice in Group 4 (Adriamycin) and 2/2 (100%) of the examined mice in Group 5 (Adriamycin + EGF-GEN). Most likely caused by the Adriamycin.

**2. Incidental findings:**

- a. Heart, dystrophic epicardial mineralization and fibrosis, multifocal, mild to moderate. Present in :  
1/2 (50%) of mice in Group 3 (Cytoxin + EGF-Gen),  
1/2 (50%) of mice in Group 4 (Adriamycin),  
1/2 (50%) of mice in Group 5 (Adriamycin + EGF-Gen),  
1/2 (50%) of mice in Group 6 (Methotrexate),  
1/2 (50%) of mice in Group 7 (Methotrexate + EGF-Gen).
- b. Ovary, ovarian tumor, (probable granulosa cell tumor). Present in 1/1 (100%) of the examined mice in Group 1 (Taxol).
- c. Thymus, thymic cysts, multifocal. Most likely are developmental. Present in 1/1 (100%) of the examined mice in Group 1 (Taxol).

**C. COMMENTS:**

Based on the results of the histologic evaluation of the test animals, it is concluded that EGF-GEN is non-toxic under the conditions of this study.

**Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.**

TABLE 2: Histopathologic Results from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.							
GROUP:	1	2	3	4	5	6	7
TREATMENT:	TXL	TXL + EGF-GEN	CTX + EGF-GEN	ADM	ADM + EGF-GEN	MTX	MTX + EGF-GEN
<b>TISSUE / DIAGNOSIS / MODIFIER(S):</b>							
<b>BONE &amp; BONE MARROW:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Erythroid, myeloid and megakaryocytic depletion, marked.	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
<b>BRAIN:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>GUT:</b>							
<b>LARGE INTESTINE:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>SMALL INTESTINE:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
<b>STOMACH:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Gastric ulcer, focally extensive, acute, with mild suppurative submucosal inflammation.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>HEART:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Dystrophic mineralization and fibrosis, epicardium, multifocal, mild to moderate.	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
<b>KIDNEY:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Tubular necrosis, acute, mild, multifocal, renal cortex.	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
<b>LIVER:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Hepatic necrosis, mild, multifocal, acute.	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)
<b>LUNG:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)

**Histopathologic Evaluation of Tissues from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.**

**TABLE 2: Histopathologic Results from SCID Mice on a Chemotherapy + EGF-GEN IP Toxicity Study. Experiment Date: 4/19/99.**

GROUP:	1	2	3	4	5	6	7
TREATMENT:	TXL	TXL + EGF-GEN	CTX + EGF-GEN	ADM	ADM + EGF-GEN	MTX	MTX + EGF-GEN
<b>TISSUE / DIAGNOSIS / MODIFIER(S):</b>							
<b>LYMPH NODE:</b>							
1. WNL	1/1 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/1 (100%)
2. NE	1/2 (50%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/2 (50%)
<b>OVARIES:</b>							
1. WNL	0/2 (0%)	1/1 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	1/1 (100%)	2/2 (100%)
2. NE	1/2 (50%)	1/2 (50%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	1/2 (50%)	0/2 (0%)
3. Follicular atrophy, moderate to marked.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/1 (100%)	1/1 (100%)	0/2 (0%)	0/2 (0%)
4. Ovarian tumor, probable granulosa cell tumor.	1/1 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>PANCREAS:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>SKELETAL MUSCLE:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>SKIN:</b>							
1. WNL	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>SPINAL CORD:</b>							
1. WNL	1/1 (100%)	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	1/2 (50%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>SPLEEN:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
3. Reduced hematopoiesis, red pulp, moderate.	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/1 (100%)	2/2 (100%)	0/2 (0%)	0/2 (0%)
<b>THYMUS:</b>							
1. WNL	0/2 (0%)	1/1 (100%)	2/2 (100%)	0/2 (0%)	1/1 (100%)	2/2 (100%)	1/1 (100%)
2. NE	1/2 (50%)	1/2 (50%)	0/2 (0%)	2/2 (100%)	1/2 (50%)	0/2 (0%)	1/2 (50%)
3. Cysts, multifocal, mild, (developmental).	1/1 (100%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>URINARY BLADDER:</b>							
1. WNL	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
<b>UTERUS:</b>							
1. WNL	2/2 (100%)	2/2 (100%)	2/2 (100%)	2/2 (100%)	1/1 (100%)	2/2 (100%)	2/2 (100%)
2. NE	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	0/2 (0%)

**NOTES:**

- Normal mice may contain the following:
  - hepatocellular sinusoidal inflammation, mild, multifocal consisting of a few small foci of macrophages, neutrophils, and/or lymphocytes;
  - nonsuppurative cholangitis, mild;
  - extramedullary hematopoiesis, mild multifocal;
  - mild infiltrates of granulocytes in the gastric submucosa, particularly at the junction of the glandular and nonglandular regions;
  - mild inflammation of the mesenteric fat and/or peritoneum.
- WNL = Within Normal Limits.
- NE = Not Examined.

## Appendix IV

Proportion Surviving Tumor Free when treated with Chemotherapy Drugs in Combination with EGF-Gen administered against MDA MB 231 in SCID mice

Treatment Group	# of Mice	Proportion Surviving		Mean EFS (days)	Median EFS (days)	vs Control	vs EGF-Gen	vs Taxol	vs Cytosan	P-rank Value
		15 Days	30 Days							
Control	10	70 ± 14.5	60 ± 15.5	40 ± 15.5	26.2 ± 2.6	32	0.0218	0.6103	0.0745	0.4148
EGF-Gen	10	30 ± 14.5	0 ± 0	0 ± 0	15.8 ± 1.7	15	0.0218	0.0051	0.2367	0.0663
Taxol	10	100 ± 0	80 ± 12.6	50 ± 15.8	45.7 ± 4.0	44.5	0.6103	0.0051	0.0093	0.0801
Adriamycin	10	70 ± 14.5	10 ± 9.5	0 ± 0	20.2 ± 2.8	18	0.0745	0.2367	0.0093	0.2604
Cytosan	10	70 ± 14.5	40 ± 15.5	20 ± 12.6	29.4 ± 5.2	23.5	0.4148	0.0663	0.0801	0.2604
Taxol + EGF-Gen	10	90 ± 9.5	40 ± 15.5	10 ± 9.5	31.6 ± 4.3	27	0.2622	0.0117	0.0166	0.0357
Adriamycin + EGF-Gen	10	60 ± 15.5	40 ± 15.5	10 ± 9.5	28.0 ± 5.2	21.5	0.1731	0.0382	0.0244	0.2135
Cytosan + EGF-Gen	10	80 ± 12.6	50 ± 15.8	30 ± 14.5	32.1 ± 5.2	28.5	0.3743	0.0300	0.0745	0.0663
										0.9442
										0.9188
										0.6103

This table is based on data collected up through day 71.

Doubling tumor progression survival of Chemotherapy compounds with EGF-Gen administered against MDA MB 231 in CB17 SCID mice

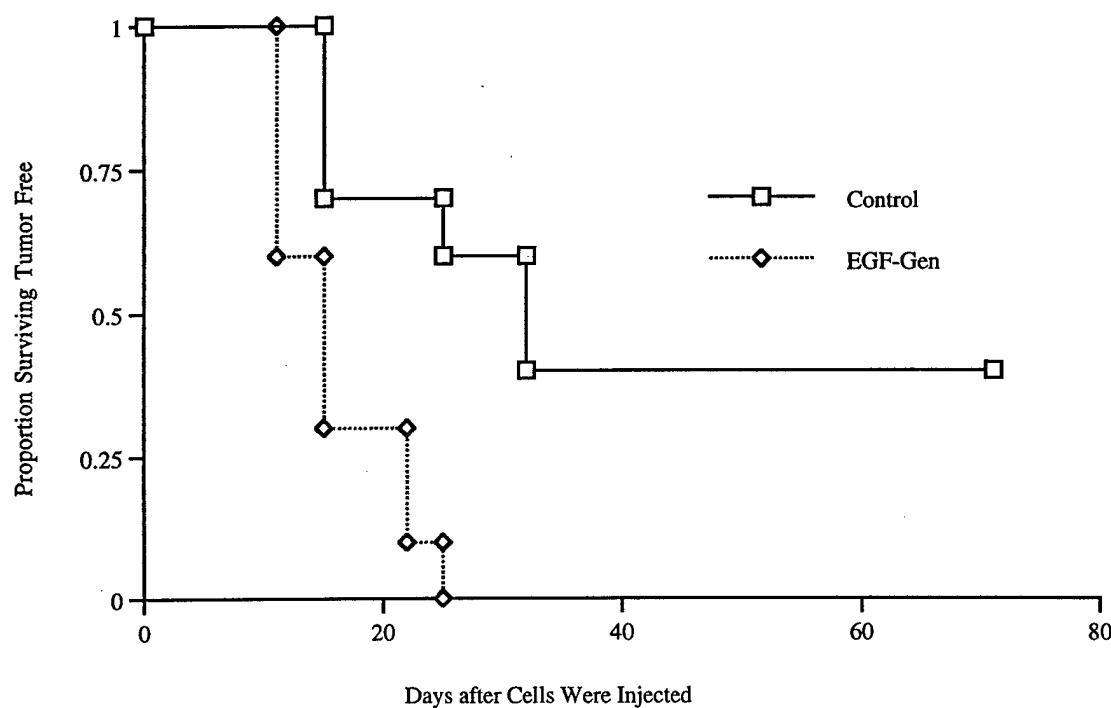
Treatment Group	# of Mice	Doubling Progression		Median PFS (days)	Mean PFS (days)	vs Control	vs EGF-Gen	vs Taxol	vs Adriamycin	vs Cytosan	vs Taxol + EGF-Gen	vs Adriamycin + EGF-Gen	vs Cytosan + EGF-Gen	P-rank Value
		30 Days	60 Days											
Control	6	50 ± 20.4	0 ± 0	30.5	34.5 ± 5.0		0.7532	0.0277	0.0747	0.0431	0.1380	0.0464	0.0464	
EGF-Gen	10	70 ± 14.5	10 ± 9.5	32	30.7 ± 2.4	0.7532		0.0166	0.0440	0.0209	0.0209	0.1097	0.0129	
Taxol	10	100 ± 0	20 ± 12.6	53	56.8 ± 2.7	0.0277	0.0166	0.3454	0.7213	0.6744	0.0330	0.0330	0.0594	
Adriamycin	9	89 ± 10.5	11 ± 10.5	53	51.3 ± 4.3	0.0747	0.0440	0.3454		0.4412	0.7794	0.2936	0.1614	
Cytosan	10	100 ± 0	40 ± 15.5	56.5	54.4 ± 4.3	0.0431	0.0209	0.7213	0.4412		0.8590	0.0858	0.3785	
Taxol + EGF-Gen	10	90 ± 9.5	30 ± 14.5	53	54.3 ± 4.5	0.1380	0.0209	0.6744	0.7794	0.8590		0.3454	0.7998	
Adriamycin + EGF-Gen	9	89 ± 10.5	0 ± 0	46	45 ± 3.9	0.0464	0.1097	0.0330	0.2936	0.0858	0.3454		0.0117	
Cytosan + EGF-Gen	10	90 ± 9.5	30 ± 14.5	56.5	55.7 ± 5.1	0.0464	0.0129	0.9534	0.1614	0.8785	0.7998	0.0117		

Tripling tumor progression survival of Chemotherapy compounds with EGF-Gen administered against MDA MB 231 in CB17 SCID mice

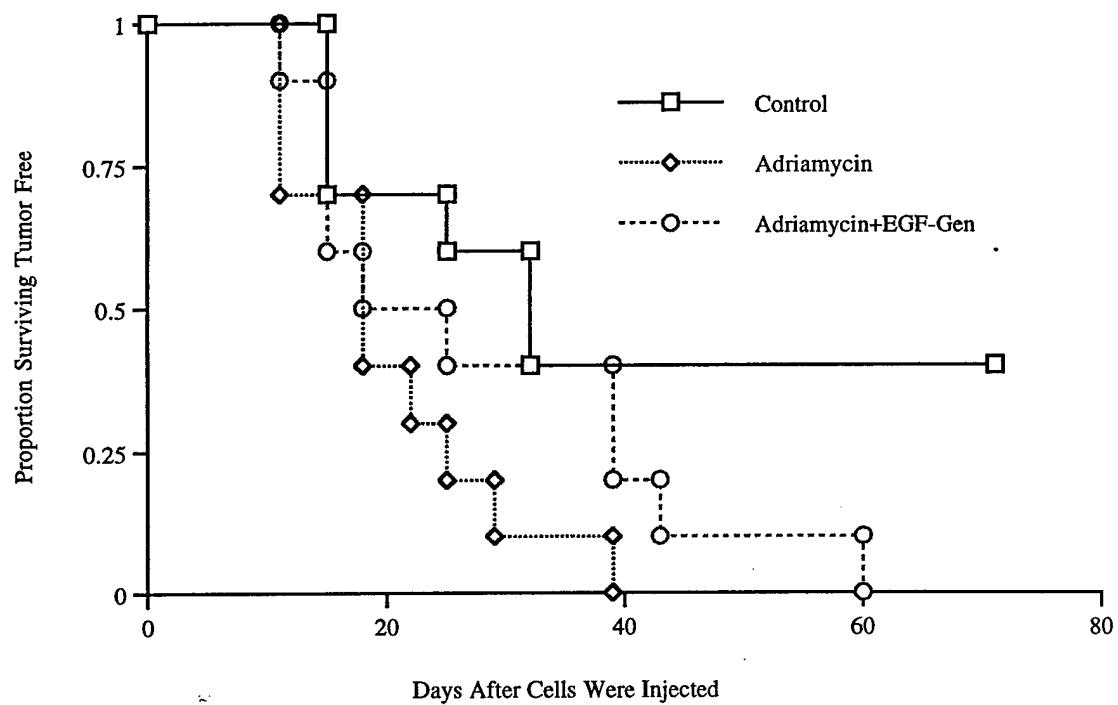
Treatment Group	# of Mice	Tripling Progression		Median PFS (days)	Mean PFS (days)	vs Control	vs EGF-Gen	vs Taxol	vs Adriamycin	vs Cytosan	vs Taxol + EGF-Gen	vs Adriamycin + EGF-Gen	vs Cytosan + EGF-Gen	P-rank Value
		30 Days	60 Days											
Control	6	83 ± 15.2	0 ± 0	34	37.5 ± 5.7		0.8927	0.0431	0.0431	0.0464	0.1159	0.0464	0.0464	
EGF-Gen	10	90 ± 9.5	10 ± 9.5	39	37.8 ± 2.0	0.8927		0.0144	0.0357	0.0059	0.0323	0.0910	0.0077	
Taxol	10	100 ± 0	30 ± 14.5	60	58.9 ± 2.6	0.0431	0.0144		0.3105	0.2135	0.6744	0.0580	0.3270	
Adriamycin	9	100 ± 0	22 ± 13.9	53	54.3 ± 3.1	0.0431	0.0357	0.3105		0.0440	0.8127	0.2626	0.0910	
Cytosan	10	100 ± 0	60 ± 15.5	67	63.3 ± 2.7	0.0464	0.0059	0.2135	0.0440		0.2340	0.0180	0.8385	
Taxol + EGF-Gen	10	100 ± 0	30 ± 14.5	56.5	57.7 ± 3.2	0.1159	0.0323	0.6744	0.8127	0.2340		0.2049	0.1834	
Adriamycin + EGF-Gen	9	100 ± 0	0 ± 0	50	50.0 ± 2.4	0.0464	0.0910	0.0580	0.2626	0.0180	0.2049		0.0117	
Cytosan + EGF-Gen	10	100 ± 0	50 ± 15.8	63.5	61.8 ± 2.1	0.0464	0.0077	0.3270	0.0910	0.8385	0.1834	0.0117		

This table is based on data collected up through day 71.

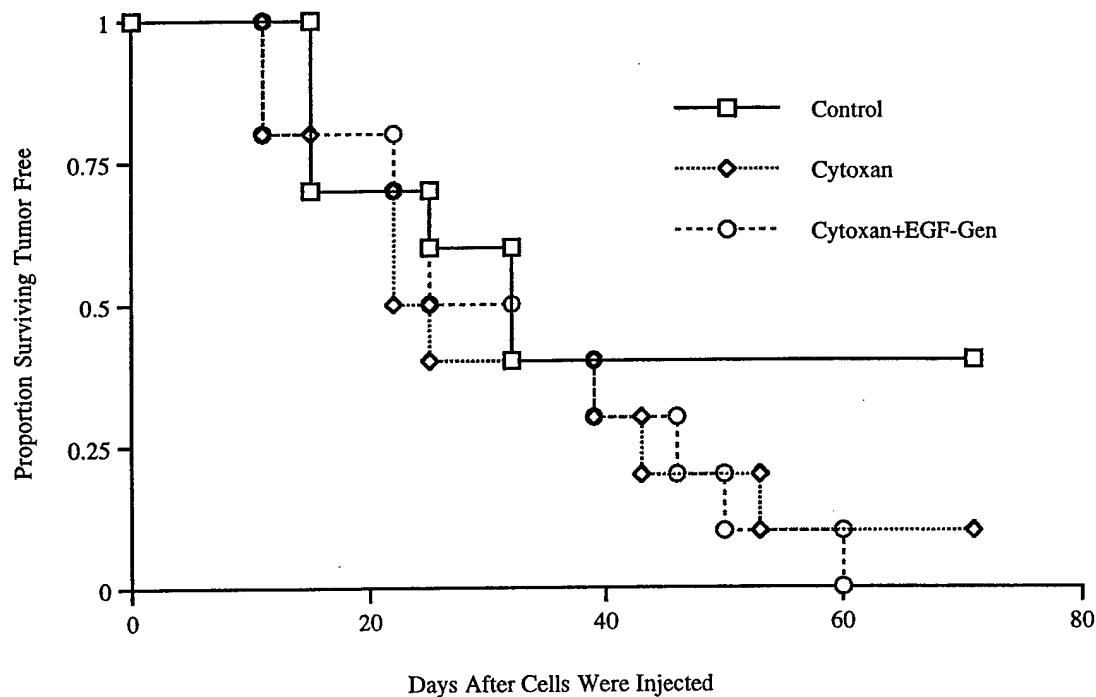
5/24/99 MDA Tumor Free Survival EGF-Gen Data



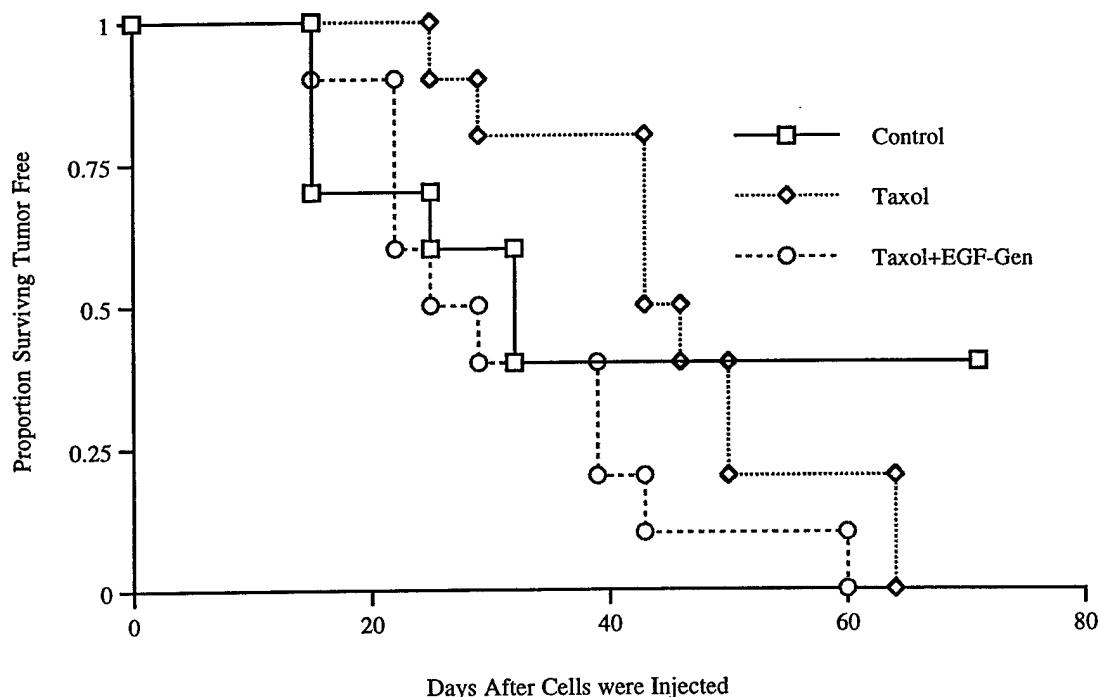
5/24/99 MDA Tumor Free Survival Adriamycin Data



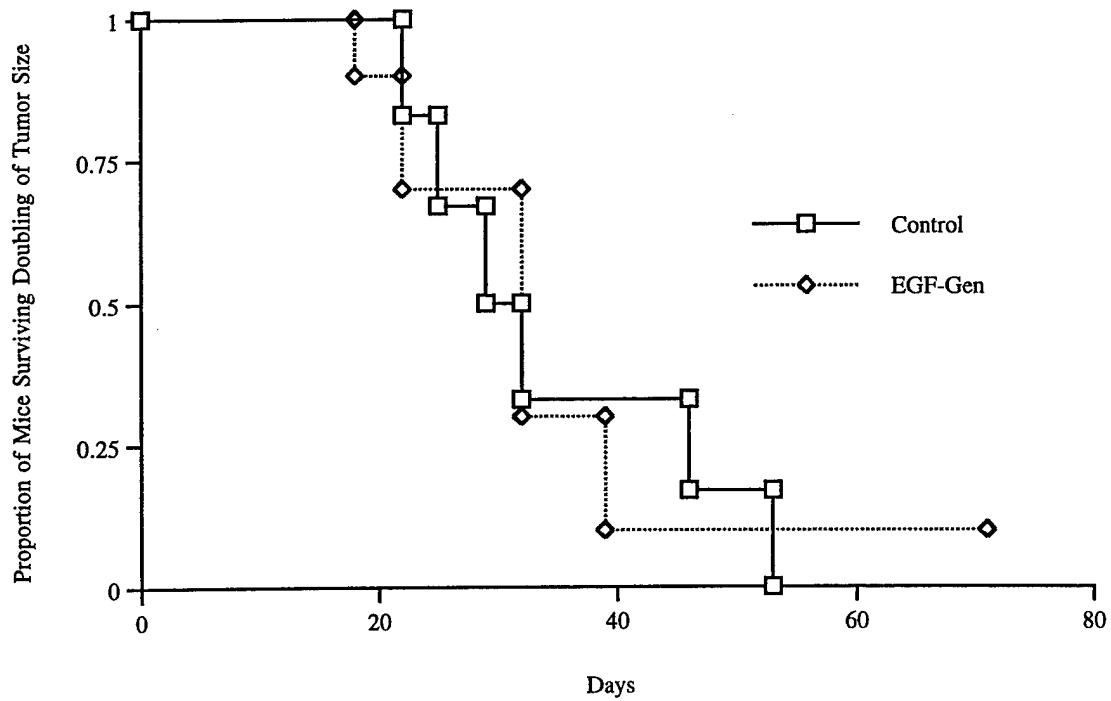
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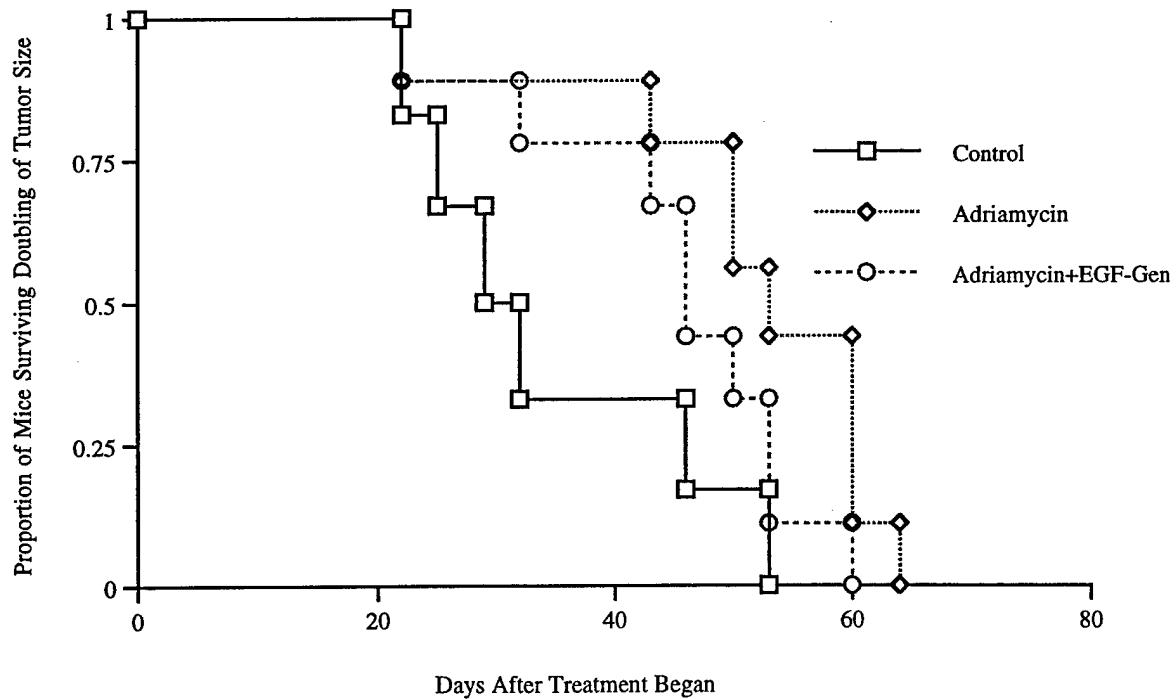
5/24/99 MDA Tumor Free Survival Taxol Data



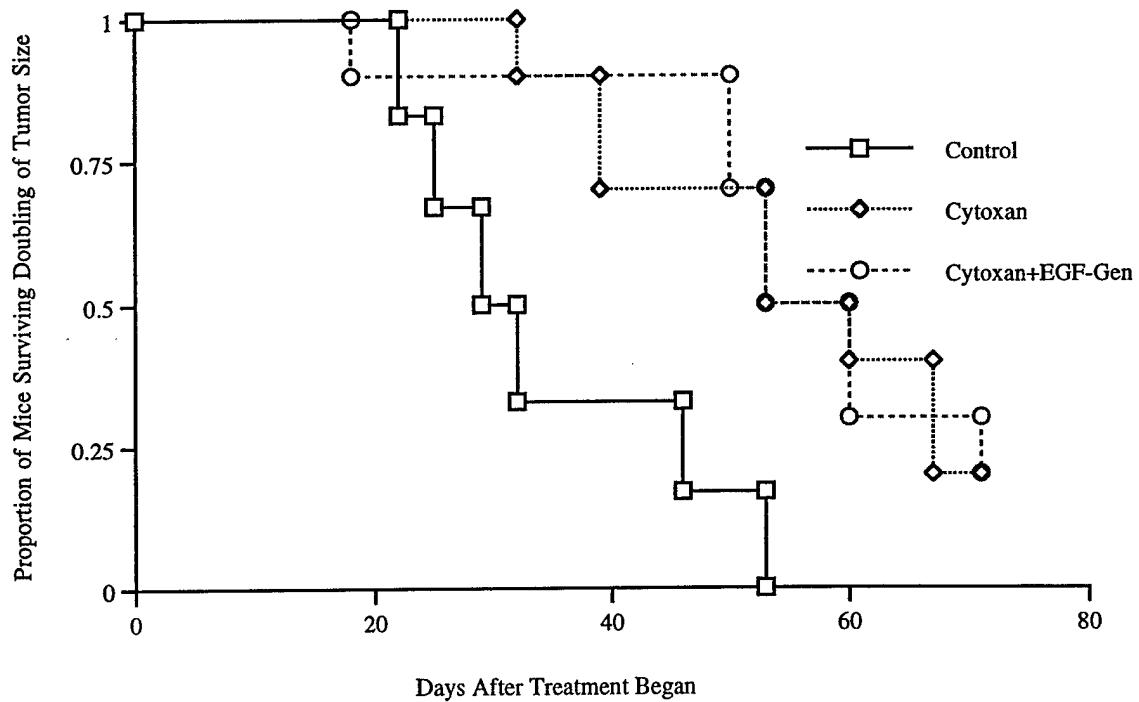
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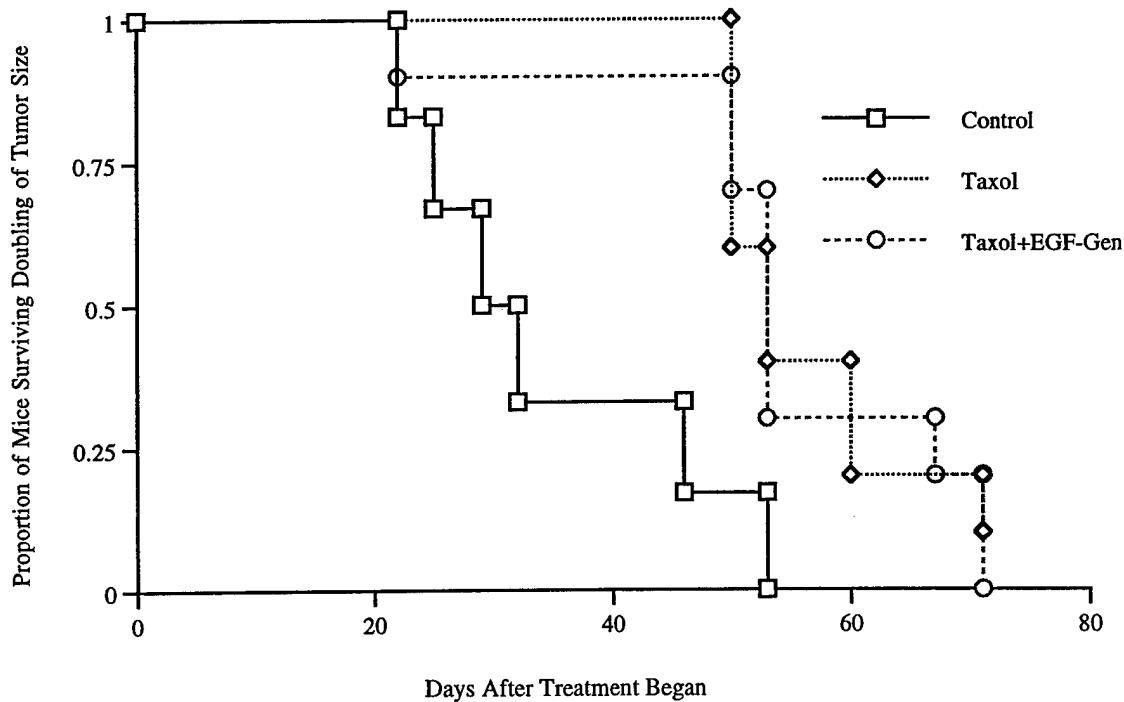
5/24/99 MDA Tumor Progression Adriamycin Doubling Data



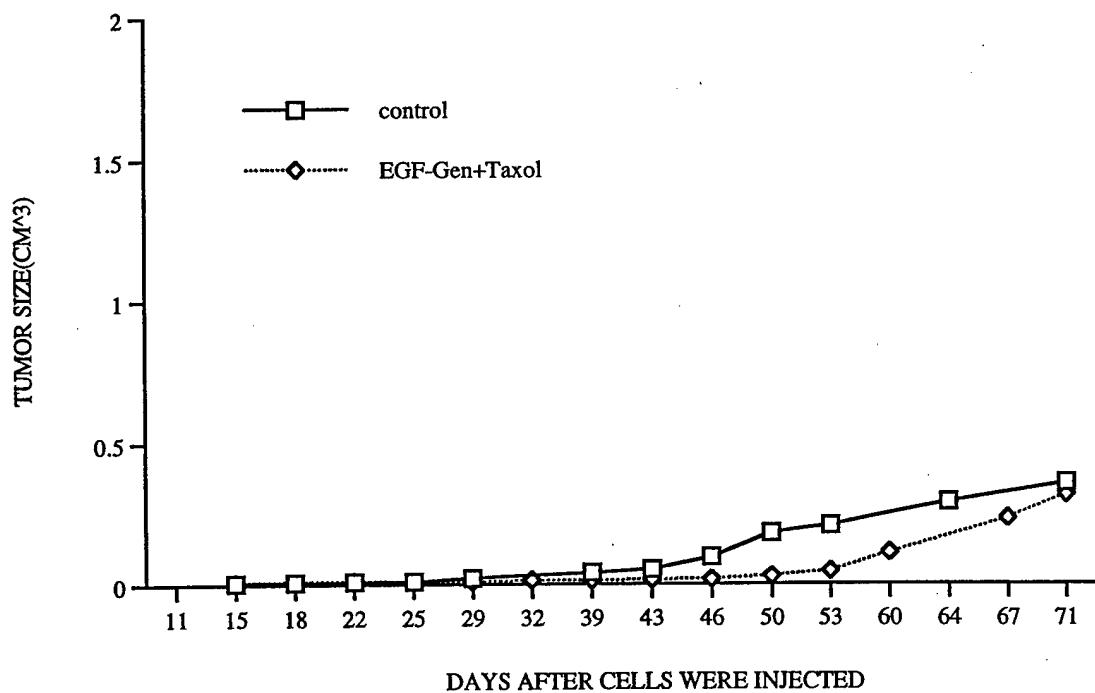
## 5/24/99 MDA Tumor Progression Cytoxin Doubling Data



5/24/99 MDA Tumor Progression Taxol Doubling Data

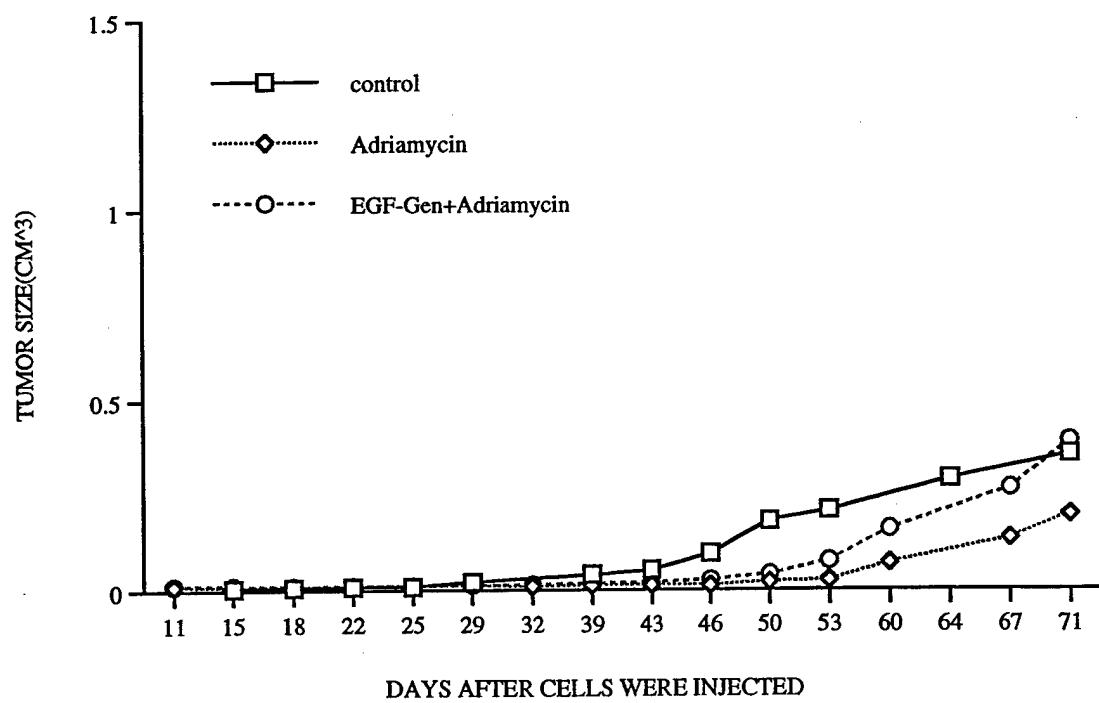


5/24/99 MDA EGF-Gen + Taxol DATA



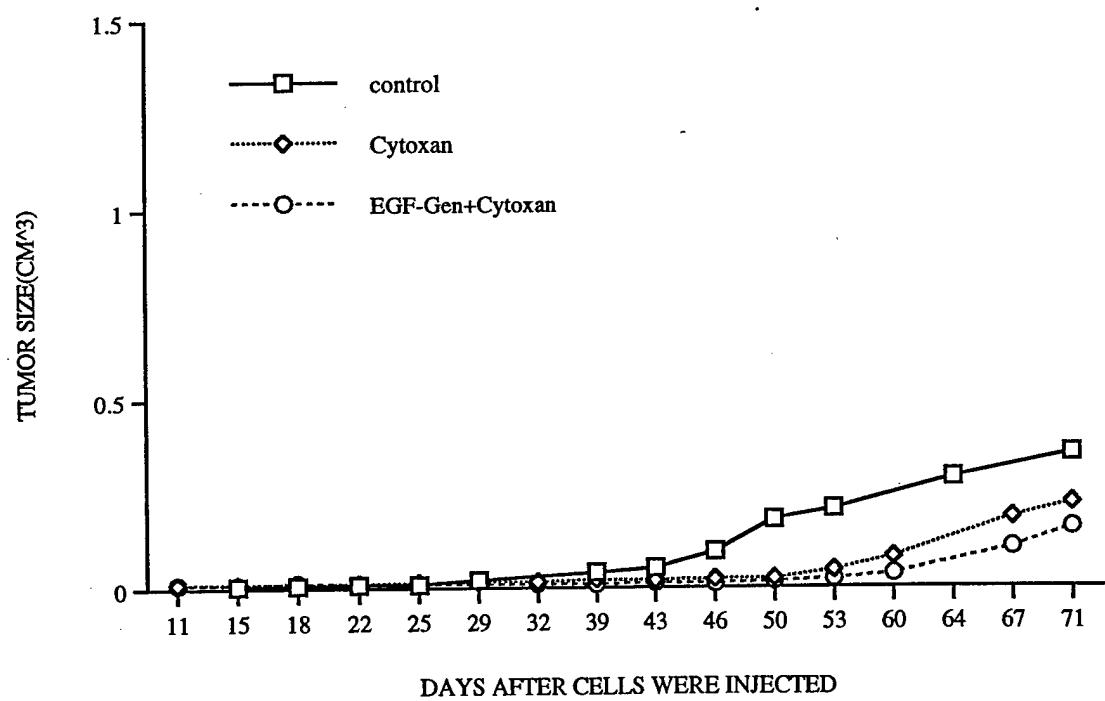
This is a graph of tumor growth in mice injected with MDA cells SQ. These mice were treated with a combination of EGF-Gen and Taxol.

5/24/99 MDA Adriamycin DATA



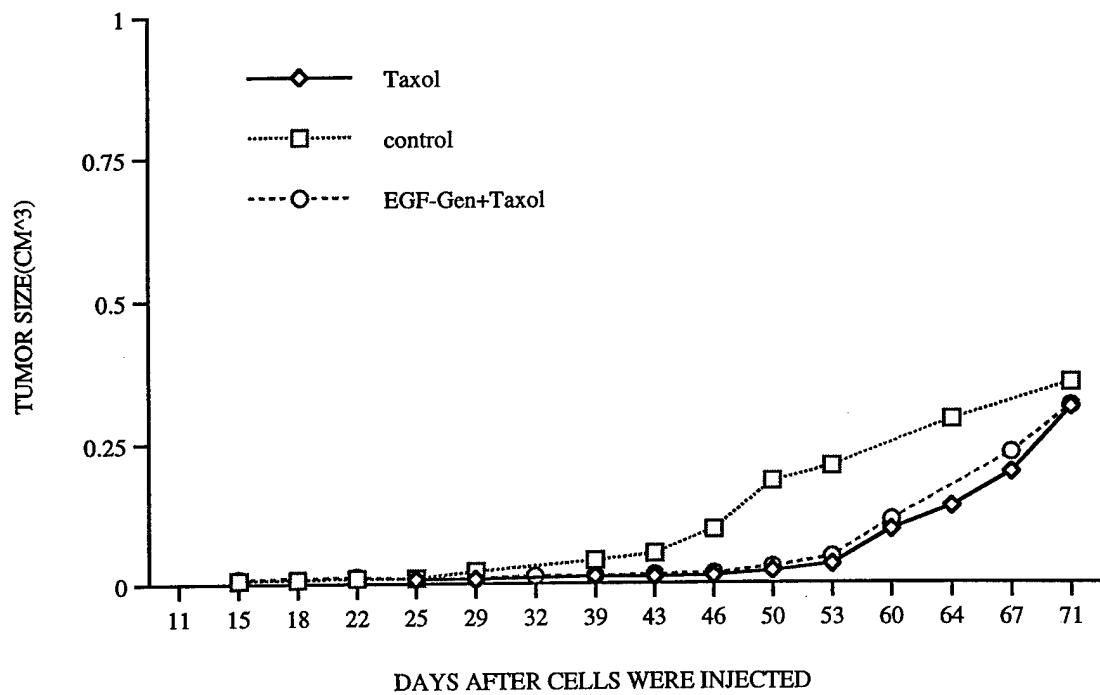
This is a graph of tumor growth in mice injected with MDA cells SQ. These mice were treated with Adriamycin on day 2.

5/24/99 MDA Cytoxin DATA



This is a graph of tumor growth in mice injected with MDA cells SQ.  
These mice were treated with Cytoxin on days 2 and 3.

5/24/99 MDA Taxol DATA



This is a graph of tumor growth in mice injected with MDA cells SQ.  
These mice were treated with Taxol on days 2-4.

## **DOCUMENT 4. Year 2000 Annual Report**

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## INTRODUCTION

During the last budget period, a systematic effort has been made aimed at identifying active Sulfa-SANPAH-linked EGF-Gen conjugates with specific HPLC elution profiles (see **Document 4**). two major species have been subjected to detailed biological testing. neither species exhibited a potent cytotoxicity against human breast cancer cells. Therefore, no animal studies have been conducted with these conjugates.

## **Materials and Methods -**

**Preparation of EGF-Genistein and Related Conjugates .** rhEGF was produced in *E. coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies and the mature protein was recovered by trypsin-cleavage followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of  $4.6 \pm 0.2$ . The endotoxin level was 0.17 EU/mg.

The recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) was initially employed in the synthesis of the EGF-Genistein(Gen) conjugates. Sulfo-SANPAH was dissolved in DMSO and used to modify EGF at a molar ratio of 1:10, EGF to crosslinker. Following size-exclusion chromatography to remove unreacted crosslinker and small molecular weight reaction products, the modified rhEGF was mixed with a 10:1 or 20:1 molar ratio of Gen (LC Laboratories, Woburn, MA) [50 mM solution in dimethyl sulfoxide (DMSO)] and then irradiated for 60 min with long-wave UV light ( 366 nm, Model UVGL-15 Multiband UV- 254/366 nm Mineralight; UVP, Upland, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a molar excess of Genistein resulted in the attachment of Gen to lysine 28, lysine 48, or the N-terminal residue of EGF. Excess Gen in the reaction mixture was removed by passage through a G25-Sephadex prepacked column.

The EGF- Gen conjugate was subsequently filter-sterilized and the protein concentration determined using the Bicinchoninic Acid(BCA) Protein Assay kit obtained from Sigma Chemical Company. Bicinchoninic acid is a chromogenic reagent, highly specific for Cu(I), which forms a purple

complex with an absorbance at 562 nm that is directly proportional to the protein concentration.

In addition to Sulfo-SANPAH, we used the following crosslinking agent obtained from Molecular BioSciences(Boulder, CO): N-5-azido-2-nitrobenzoyloxysuccinimide(ANB-NOS), with a chain length of 7.7 Å compared to 18.2 Å for Sulfo-SANPAH. ANB-NOS also has a phenyl azide at one end to react with Genistein following photolysis and an N-hydroxysuccinimide ester at the other end to react with protein amino groups.

**HPLC Analysis.** Reverse phase HPLC using a Hewlett-Packard (HP) 1100 series HPLC instrument was used to monitor and characterize the EGF-Gen conjugations. Analytical HPLC was performed using a LiChrospher 100(RP-18, 5 um) reverse phase column (250x4 mm, Hewlett-Packard). HPLC chromatograms were run at wavelengths of 280 nm, 325 nm, or 480 nm using the multiple wavelength detector option supplied with the instrument. UV spectra were generated for the individual peaks of interest in the chromatogram. Five - 100 uL samples were applied to the above column and analysis was achieved using a gradient flow as follows: t = 0, 20% D; t = 5, 30% D; t = 9, 38% D; t = 20, 43.5% D; t = 35, 100% D; t = 50, 100% D; t = 55, 20% D; t = 56 stop. Eluent C consisted of a mixture of 0.1% trifluoroacetic acid(TFA) in water and eluent D contained 80% acetonitrile (CH<sub>3</sub>CN), 20% H<sub>2</sub>O, and 0.1% TFA.

Size-exclusion chromatography was carried out using a Beckman System Gold Instrument equipped with either a preparative TSKG3000SW column equilibrated in 100 mM sodium phosphate buffer, pH 6.8 at a flow rate of 3 mL/minute or an analytical TSKG3000PW column run in the same buffer at a flow rate of 0.2 mL/min

**Mass Spectrometry.** Mass spectrometric analysis was routinely performed to determine the relative molecular weights of the modified EGF and EGF-

Genistein conjugates using a Hewlett-Packard Model G2025A matrix-assisted laser desorption/ionization mass spectrometer with linear time-of-flight mode (MALDI-TOF). In conjunction with the Hewlett-Packard instrument were a sample preparation assembly model G2024A including a high vacuum pump and a Dos-Chem station controller model G1030A. Before starting the experiment, the instrument was calibrated with protein standards G2025A supplied by Hewlett-Packard; mass calibration was used by peak centroiding at the 80% level. Sinnapinic acid(Hewlett-Packard) was used as a matrix source. Samples were prepared by spotting 1 uL of a mixture of protein, in phosphate buffer, with the matrix solution(1:1, v/v) on the gold surface of the probe with subsequent evaporation under vacuum. Ionization was accomplished with a laser radiating at a 337-nm wavelength(5 ns pulses, laser energy 1.97 uJ) in both single shot and multiple shot modes. The analyzer was used in the linear mode at an accelerating voltage of 28 kV. The obtained spectra represent the sum of consecutive laser shots and have not been smoothed.

**Zymography Procedure.** 500,000 cells were plated into each well of a 6 - well microtiter plate and incubated overnight in 2 mL of complete medium for cell attachment. Following the overnight incubation, cells were washed 3 times in serum-free medium containing 1% penicillin-streptomycin(Pen - Strep). Two mL of serum-free medium plus Pen - Strep + the test compounds were added and the cells incubated at 37° C for the desired period of time.

Following the incubation period(usually 24 - 96 hr), the cell supernatants were harvested and centrifuged 10 minutes to remove cell debris. The supernatants were then concentrated using centrifugal concentrating devices(Centricon, Millipore) with a 30 kDa molecular weight cut-off. Protein concentrations were determined using the BCA assay and equal amounts of protein were run on 10% SDS - PAGE gels containing 0.1%

gelatin(Sigma, Type A from porcine skin, approximately 300 bloom).

Samples were not reduced or heated for the gels.

After electrophoresis, the gels were rocked at room temperature in 2.5% Triton X-100 for 30 minutes, followed by rocking in Developing Buffer(10 mM Tris base, 40 mM Tris - HCl, 200 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.02% Brij 35) for 60 minutes at room temperature. Fresh Developing Buffer was added to the gels and they were incubated overnight at 37° C. The Buffer was decanted and the gels stained with gentle rocking for a minimum of 60 minutes in 0.5% Coomassie Blue. Gels were destained in 10% methanol/5% acetic acid until clear bands became visible. Gels were stored in water until photographed and dried. This procedure was from Yiwei L, Bhuiyan M, Alhasan S, Senderowicz AM, and Sarkar FH. Induction of Apoptosis and Inhibition of c-erbB - 2 in Breast Cancer Cells by Flavopiridol. *Clinical Cancer Research* 5:223 - 229(2000).

**SDS-PAGE Analysis.** SDS-PAGE was used to monitor the preparation and purification of the EGF-Genistein conjugates. 10 - 20% tris tricine gradient gels (BioRad Laboratories) were stained with GelCode Blue to visualize the protein bands.

**Human Foreskin Fibroblast Cell Line.** Hs 27(ATCC CRL - 1634) is one of a series of human foreskin fibroblast lines developed at the Naval Biosciences Laboratory(NBL) in Oakland, CA. This cell line has the normal male karyotype, 46XY.

**Breast Cancer Cells.** MDA-MB-231 (ATCC HTB-26) is an EGF-R positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51 year old patient. BT-20 (ATCC HTB-19) is another EGF-R positive breast cancer cell line isolated from the primary breast tumor of a 74 year old patient with grade II mammary adenocarcinoma. SQ-20B is a squamous cell carcinoma of the head and neck.

MDA-MB-231 cells are cultured in Leibovitz's L-15 medium plus glutamine; BT-20 breast cancer cells are maintained in MEM medium containing 0.1 mM NEAA and Earle's BSS; SQ-20B cells are in DMEM. All media are further supplemented with 10 % fetal bovine serum(DMEM contains 20% FBS, not heat-inactivated). For subculturing, medium is removed from the flasks containing a confluent layer of cells and fresh 0.25% trypsin added for 1-2 min. Trypsin is removed and cultures incubated for 5-10 min at 37°C until the cells detached. Fresh medium is then added and the cells aspirated and dispensed into new flasks.

**Cytotoxic Activity of EGF-Genistein.** The specific cytotoxic activity of the EGF-Genistein conjugates was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim Corp., Indianapolis, IN). Briefly, exponentially growing breast cancer cells were seeded into a 96-well plate at a density of  $2.0 \times 10^4$  cells/well and incubated for 18 - 24 hr at 37°C prior to drug exposure. On the day of treatment, culture medium was carefully aspirated from the wells and replaced with fresh medium containing the EGF-Genistein conjugates or unconjugated EGF. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 48 - 72 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere( MDA-MB-231 cells were incubated in the absence of CO<sub>2</sub>). To each well, 10 µl of MTT (0.5 mg/ml final concentration) was added and the plates incubated at 37°C for 4 hours to allow MTT to form formazan crystals by reacting with metabolically active cells . The formazan crystals were solubilized for a minimum of 4 hr at 37°C in a solution containing 10% SDS in 0.01 M HCl. The absorbance of each well was measured in a microplate reader (Labsystems) at 540 nm. The absorbance was a measure of cell viability; the greater the absorbance the greater the cell viability.

**EGF Stimulation/Viability Assays.** Cell suspensions of CRL - 1634 and MDA - MB - 231 were prepared in serum-free DMEM at a final cell concentration of  $4 \times 10^4$  cells/mL. Each sample tube contained 1 mL of the cell suspension, 80 uL of FBS, 0 - 40 uL of EGF, and DMEM to a final volume of 4 mL. The contents of the tubes were gently mixed and 1 mL amounts added to each of 4 wells of a 24-well plate. The final concentration in each well was  $1 \times 10^4$  cells/mL in DMEM/2% FBS  $\pm$  EGF or EGF-conjugates. The cells were refed on day 3 and counted on day 5.

**Colony Assays.** After overnight treatment with EGF-Gen or PBS, cells were resuspended in clonogenic medium consisting of alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50  $\mu$ M 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/mL/dish and cultured in a humidified 5% CO<sub>2</sub> incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as % inhibition of clonogenic cells at a particular concentration of the test agent using the formula: % Inhibition = (1 - Mean # of colonies [Test] / Mean # of colonies [Control]) x 100.

## **Results -**

Our initial EGF-Genistein conjugates were formed using Sulfo-SANPAH as the photolabile crosslinker. EGF was modified using a 10:1 molar ratio of Sulfo-SANPAH : EGF followed by 60 minutes of photolysis in the presence of longwave UV and a 10 - 20-fold molar excess of Genistein. Size-exclusion HPLC revealed the presence of high-molecular weight material and SDS - PAGE showed the presence of EGF multimers.

Photolyzing the highly SANPAH-modified EGF at high protein concentrations appeared to be causing the formation of EGF-EGF multimers so we tried photolyzing the Sulfo-SANPAH - Genistein mixture(in DMSO) prior to the addition of the EGF. This "pre - photolysis" mixture contained a 10:1 or 2.5:1 molar excess of Genistein to crosslinker in order to increase the opportunity for the active nitrene to link to Genistein rather than to another SANPAH. Further chemical analyses of this photolyzed mixture indicated the possible formation of a Genistein - Sulfo-SANPAH ester. Since it was felt that this ester would probably be unstable during subsequent conjugation and storage conditions, we generated future EGF-Genistein conjugates using the original published procedure.

Reverse-phase HPLC analysis was performed on EGF- Genistein conjugates prepared using a 10:1 ratio of the Sulfo - SANPAH crosslinker to EGF. **Figure 1(A - E)** shows HPLC patterns for the SANPAH-modified EGF itself(in the absence of Genistein), the EGF-Genistein conjugate formed using a 20:1 ratio of Genistein to EGF, and EGF, Sulfo-SANPAH, and Genistein controls. **Figure 1F** shows the pattern for the EGF-Gen conjugate formed when a 10:1 ratio of Genistein was added to the SANPAH-modified EGF.

In this series of experiments, native, monomeric EGF has a retention time between 13 and 14 minutes and is detected at a wavelength of 280 nm. There is no free EGF present in any of the EGF conjugates; we believe the peak eluting between 44 and 46 min may represent denatured,

aggregated EGF caused by the reverse-phase column conditions. All of the HPLC traces show a number of peaks which are detectable at 280 and 480 nm. The UV spectra of these peaks reveal the absorbance peak at 280 nm(characteristic of EGF) as well as an absorbance at 480 nm indicating the presence of the SANPAH moiety.

When Genistein has been added to the conjugation mixture(**Figure 1**), the presence of unreacted Genistein, with a retention time of 19 - 20 minutes, can be detected at a wavelength of 280 nm in this reverse-phase system. The UV spectrum is characteristic with a shoulder at 330 nm; UV spectra of potential EGF-Genisten conjugates, eluting at 36 - 38 minutes, possess this shoulder along with an absorbance at 480 nm.

We then used this reverse-phase HPLC method to isolate enough material eluting at approximately 30 and 38 minutes(the 2 major peaks of interest present in EGF-Genistein) to test in viability and MTT assays. EGF itself was run through the HPLC as a control. The fractions isolated from the HPLC runs were evaporated under nitrogen to remove the acetonitrile. 10X PBS was added to neutralize the samples and bring them to the usual 1X PBS buffer conditions.

**Figure 2A** shows the results of an EGF Growth/Stimulation Assay using the CRL-1634 cell line and various concentrations of stock EGF. We chose a concentration of 2.5 ng/mL for the final concentration of EGF in subsequent assays using this cell line since we saw maximum stimulation at this dose. We also used this assay to test the effect of various concentrations of DMSO on the ability of EGF to stimulate these cells since Genistein and the crosslinkers are very insoluble in water at the concentrations we are using(**Figure 2B**). Final concentrations of DMSO are 5% for the Sulfo-SANPAH modification and 10 - 20% for the Genistein step and it is clear that these amounts are inhibitory when mixed with 2.5 mg/mL EGF, in PBS, for 2.5 hr at room temperature with gentle rocking. **Figure 3** shows that HPLC-purified EGF is still able to stimulate CRL-1634 cells. Similar experiments were conducted using MDA-MB-231 breast cancer

cells(**Figure 4**). In this case, EGF did not stimulate the cells to the same extent that CRL-1634 cells were stimulated and some inhibition of growth was seen when cells were incubated with EGF that had passed through the HPLC column.

**Figures 5A and 5B** show results of the EGF Growth Assay using CRL-1634 incubated in the presence of the parent EGF-Gen conjugate as well as fractions isolated from the reverse-phase HPLC. **Figures 6A and 6B** show a similar experiment using MDA-MB-231 breast cancer cells. Although the parent EGF-Gen conjugate appeared to stimulate both cell lines at a concentration of 2.5 ng/mL, the 38' fraction brought this stimulation nearly to the control level(no EGF present) in the CRL-1634 cells while the MDA cells were stimulated by this fraction. The 44' peak was also present in stock EGF preparations and may represent denatured or aggregated EGF.

In previous studies we substituted shorter chain-length and less hydrophobic crosslinkers for Sulfo-SANPAH in order to reduce aggregation due to protein-protein hydrophobic interactions. The short-chain crosslinker, ANB-NOS, resulted in less precipitation/aggregation than was seen using Sulfo-SANPAH and was used in recent experiments to form EGF-ANB-NOS-Gen conjugates. EGF-ANB-NOS-Gen conjugates were prepared by photolyzing ANB-NOS-modified EGF for one hr under longwave UV in the presence of excess Genistein. **Figures 7A - 7F** show representative HPLC chromatograms of EGF-ANB-NOS and EGF-ANB-NOS-Gen conjugates. UV scans are included for the major peaks of interest and for the EGF, ANB-NOS, and Genistein standards. There appears to be no monomeric, native EGF present in the conjugates. ANB-NOS modification of EGF leads to a number of peaks with characteristics of both EGF and the ANB-NOS crosslinker. When photolysis occurs in the presence of excess Genistein, no new peaks appear, but the broad peak at 26.646 min has an additional shoulder detected in the UV scan.

The ANB-NOS-EGF and EGF-ANB-NOS-Gen conjugates were tested against CRL-1634 and MDA-MB-231 cells in the stimulation/growth assay

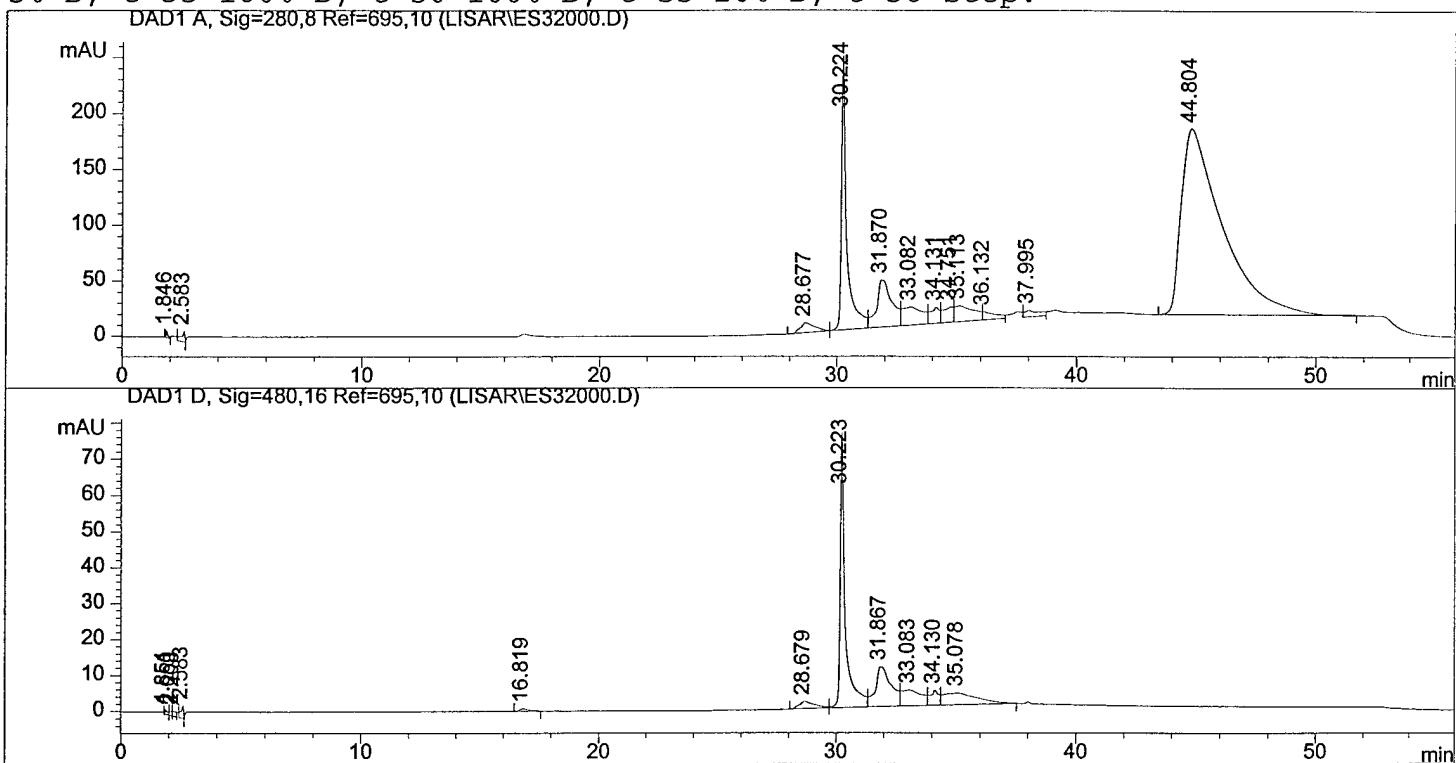
previously described. **Figures 8A and 8B** show the results from one such assay. The CRL-1634 cells were stimulated at 2.5 ng/mL concentrations of all the test compounds, the conjugates stimulated more than the stock EGF. A concentration of 10 ng/mL was used for the MDA-MB-231 cells. In this case, EGF was stimulatory but all the conjugates inhibited growth to some extent. These experiments suggest that the ANB-NOS crosslinker may generate more potent conjugates than Sulfo-SANPAH and that even ng/mL concentrations of these conjugates may show inhibition against breast cancer cell lines.

Our most recent experiments have concentrated on zymography experiments using MDA-MB-231, BT-20, and SQ-20B cell lines. We were interested in determining the species of gelatinase enzymes present in various breast cancer cell lines and to see what effect our EGF-Gen conjugates might have on their activity. Experiments were set up as described in the Materials and Methods section. Control cells received no EGF. Since Genistein was added in DMSO, we used a control which received the same amount of DMSO(1% final concentration). Cells were pulsed daily for up to 72 hr and harvested the following day. Ten to twenty-five ug amounts of concentrated supernatant protein were loaded per lane; for each gel, each lane received an equal amount of protein. **Figures 9A - 9C** show preliminary examples of gels run on these cell lines. In general, more inhibition was seen with the longer incubation times(and, therefore, higher cumulative amounts of conjugate). Genistein was very inhibitory, due in large part to the amount of DMSO present. Future experiments will use much lower Genistein concentrations. The gelatinase patterns varied in type and intensity among the different cell lines. Comparing the clear bands of enzyme activity to the molecular weight standards(clearly visible on most gels after staining with Coomassie Blue), Gelatinase A(MMP-2) may be present at 62 - 68 kDa. We would also expect to see Gelatinase B(MMP-9) (as reported in the literature for related cell lines) at approximately 92 kDa

but the upper clear band visible on our gels appears to be much larger than this relative molecular weight.. In all three cell lines tested, the EGF-Gen conjugates inhibit the gelatinase activity below the control level. The EGF-SANPAH-Gen conjugate appears to be more active than the EGF-ANB-NOS-Gen conjugate under these conditions. Incubation with EGF itself, daily concentrations of 10 ug/mL were used in these experiments, generally resulted in pronounced stimulation of gelatinase activity. These experiments are preliminary, but interesting enough to pursue further. We will use antibodies and inhibitors directed against Gelatinase A and B to determine if those are the main species present in these cell lines and to investigate the nature of the higher molecular weight band of gelatinase activity seen in our gels. We will also test our conjugates against other cell lines.

EGF/SAN (1:10) 3/20/00 2.14 mg/ml  
 LiChrospher 100 column (RP-18, 5um). Flow = 1 mL/min.

=====
 Injection Date : 3/20/00 8:36:42 PM Seq. Line : 4  
 Sample Name : EGF/SAN Vial : 4  
 Acq. Operator : Kenny Inj : 1  
 Inj Volume : 50  $\mu$ l  
 Different Inj Volume from Sequence ! Actual Inj Volume : 10  $\mu$ l  
 Sequence File : C:\HPCHEM\1\SEQUENCE\EGF32000.S  
 Method : C:\HPCHEM\1\METHODS\LISAEFG2.M  
 Last changed : 3/20/00 5:08:16 PM by Kenny  
 EGF samples with Lichrospher 100 column. C: H<sub>2</sub>O, 0.1% TFA D: 80% ACN 20%  
 H<sub>2</sub>O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43%.  
 5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



**Figure 1A** - Figure 1A shows a reverse-phase HPLC pattern of EGF-SANPAH made using a 10:1 molar ratio of SANPAH to EGF. Unmodified EGF would elute between 13 and 14 min and unreacted Sulfo-SANPAH at 25 - 26 min under these conditions.

EGF/SAN/GEN (1:10, 1:20) 17% DMSO  
1 HR LWUV 3/20/00 Fractions collected.  
LiChrospher 100 column (RP-18, 5um).

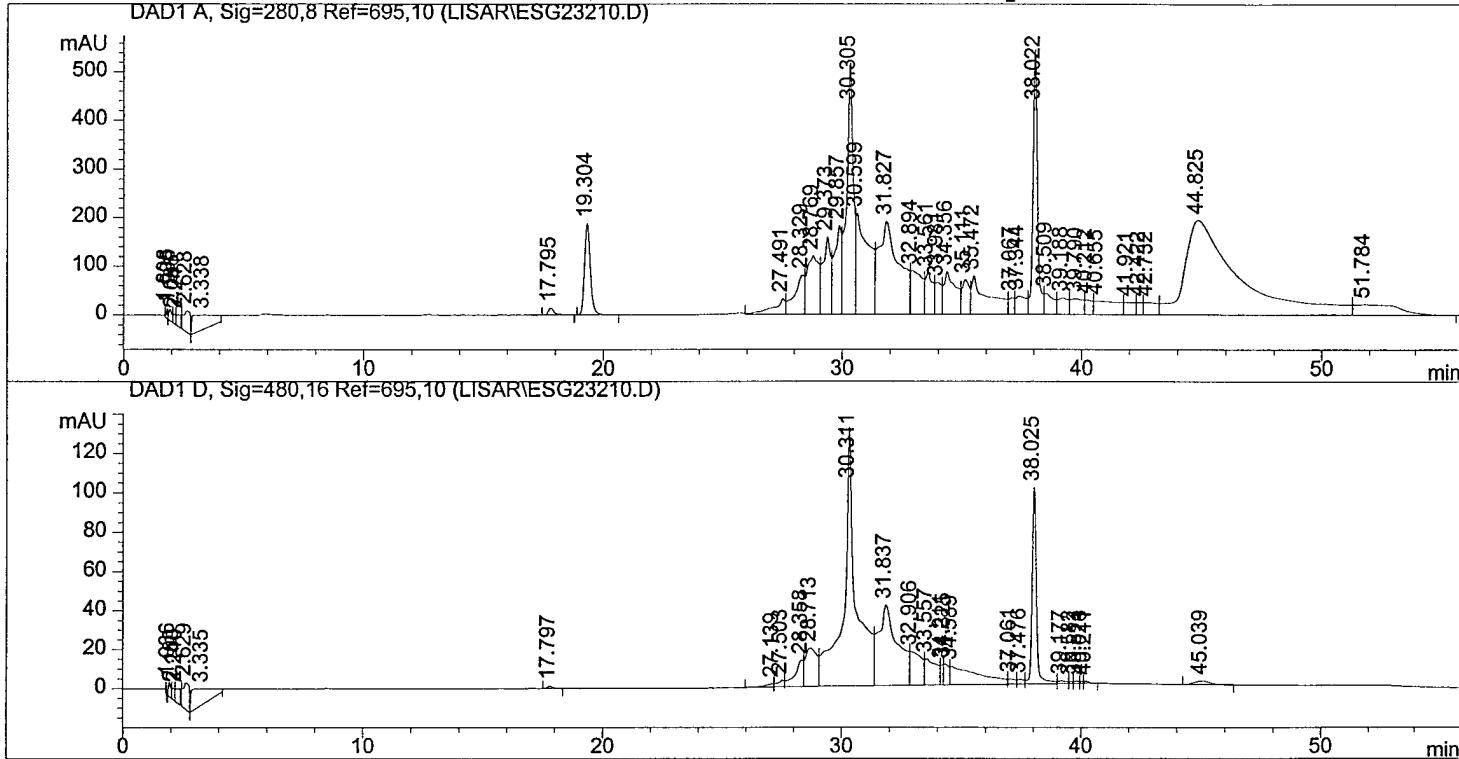
Injection Date : 3/21/00 11:45:25 AM Seq. Line : 2  
Sample Name : EGF/SAN/GEN Vial : 1  
Acq. Operator : Deb Inj : 1  
Inj Volume : 100  $\mu$ l

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Method : C:\HPCHEM\1\METHODS\LI.SAEGE2.M

Last changed : 3/21/00 10:30:53 AM by Deb

Last changed : 3/21/00 10:30:53 AM by Deb  
ECE samples with Lighrographer 100 column - C:\

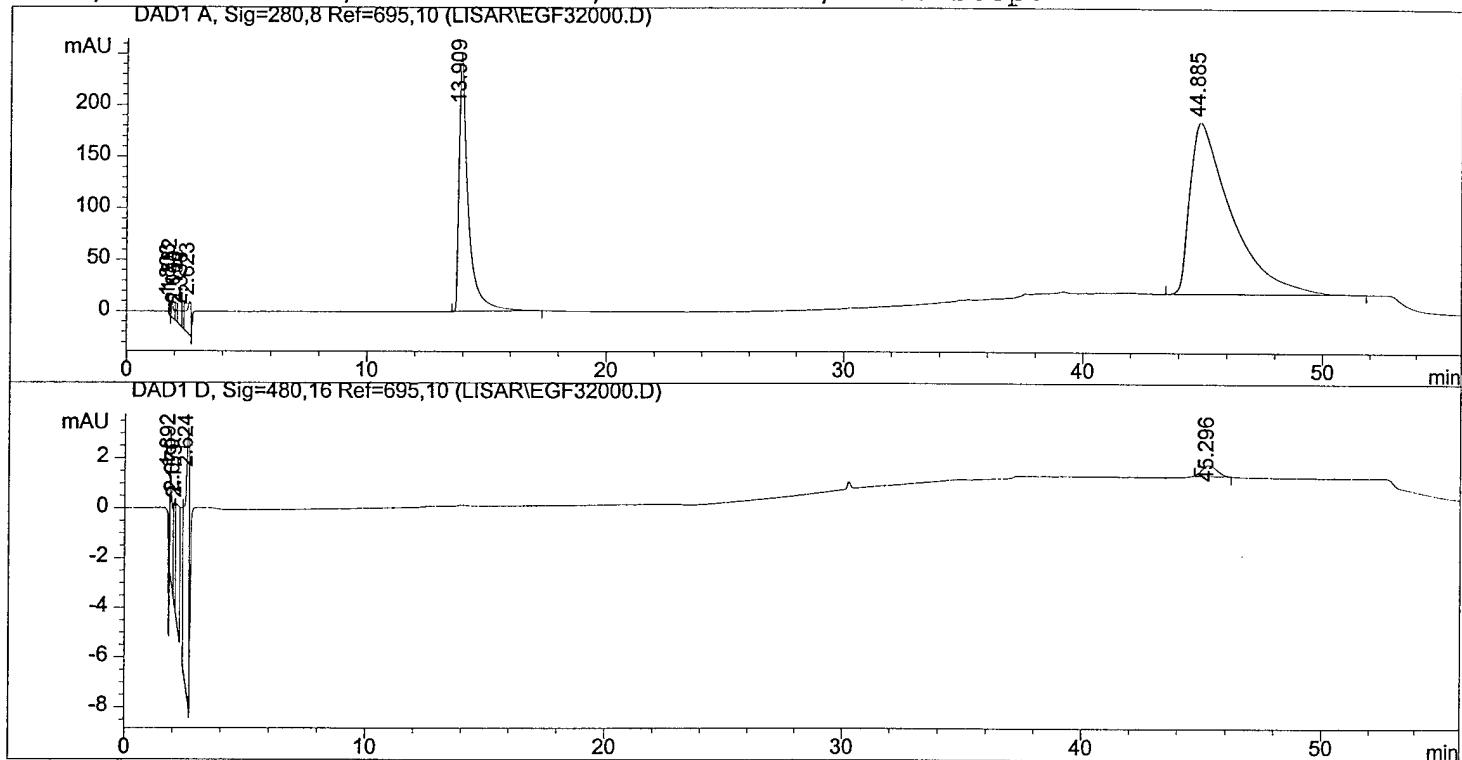
EGF samples with Lichrospher 100 column. C: H<sub>2</sub>O, 0.1% TFA D: 80% ACN 20% H<sub>2</sub>O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43% D; t=35 5% D; t=50 100% D; t=55 20% D; t=56 stop.



**Figure 1B** - Figure 1B shows the pattern for EGF - SANPAH - Gen using a 20:1 molar ratio of Genistein to EGF. The spectrum of the peak eluting at 30 min has characteristics of EGF and the SANPAH crosslinker; the peak at 38 min has characteristics of EGF, SANPAH, and Genistein. Unreacted Genistein is present (19 min).

EGF 1 mg/ml in PBS 3/20/00  
 LiChrospher 100 column (RP-18, 5um). Flow = 1 mL/min.

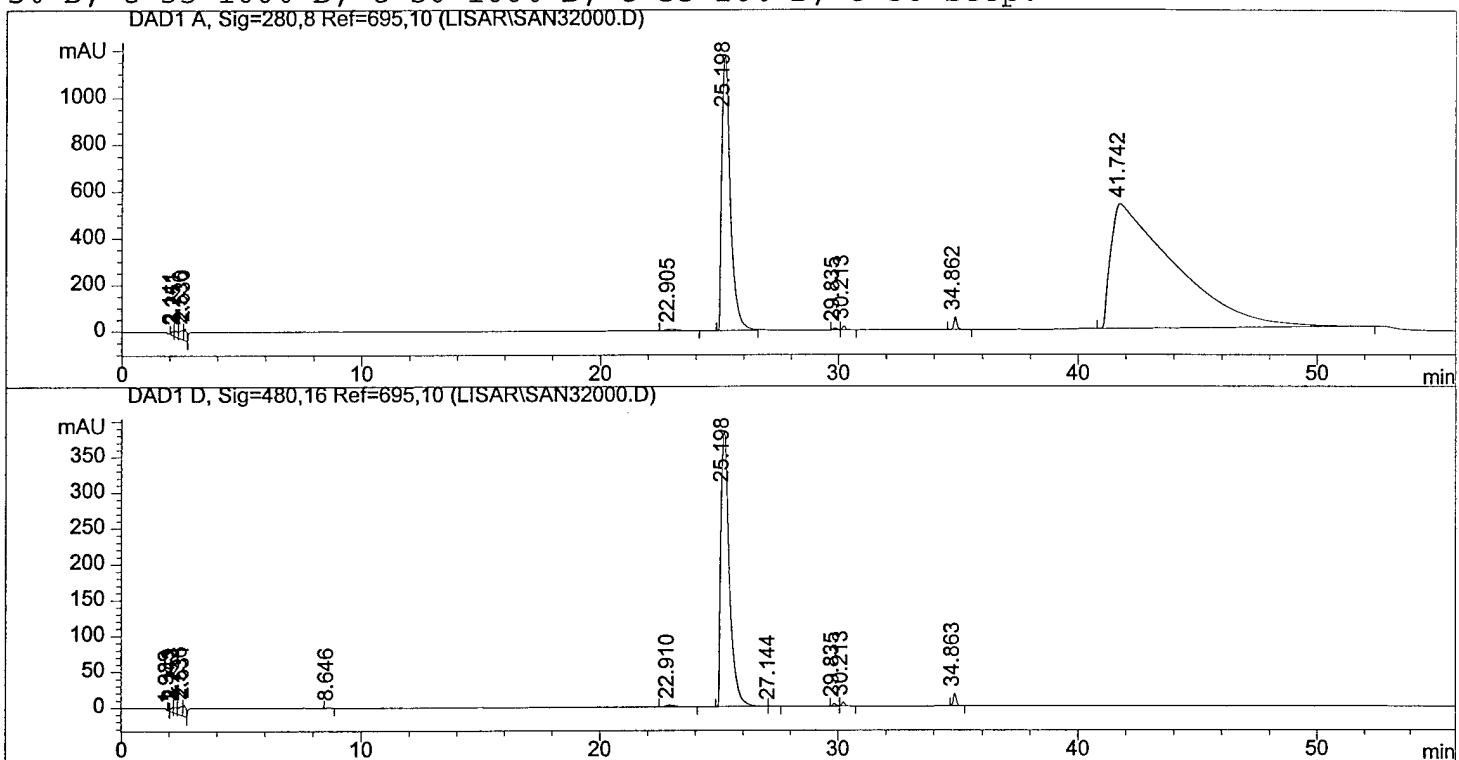
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 Injection Date : 3/20/00 11:58:09 PM Seq. Line : 7  
 Sample Name : EGF Vial : 7  
 Acq. Operator : Kenny Inj : 1  
 Inj Volume : 50  $\mu$ l  
 Sequence File : C:\HPCHEM\1\SEQUENCE\EGF32000.S  
 Method : C:\HPCHEM\1\METHODS\LISAEGF2.M  
 Last changed : 3/20/00 5:08:16 PM by Kenny  
 EGF samples with Lichrospher 100 column. C: H<sub>2</sub>O, 0.1% TFA D: 80% ACN 20%  
 H<sub>2</sub>O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43%.  
 5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



=====
 Figure 1C - Figure 1C shows the reverse-phase HPLC pattern for EGF.

Sanpah 1mg/20 ul DMSO 5 ul + 250 ul methanol.  
 LiChrospher column (RP-18, 5 um) Flow 1 ml/min

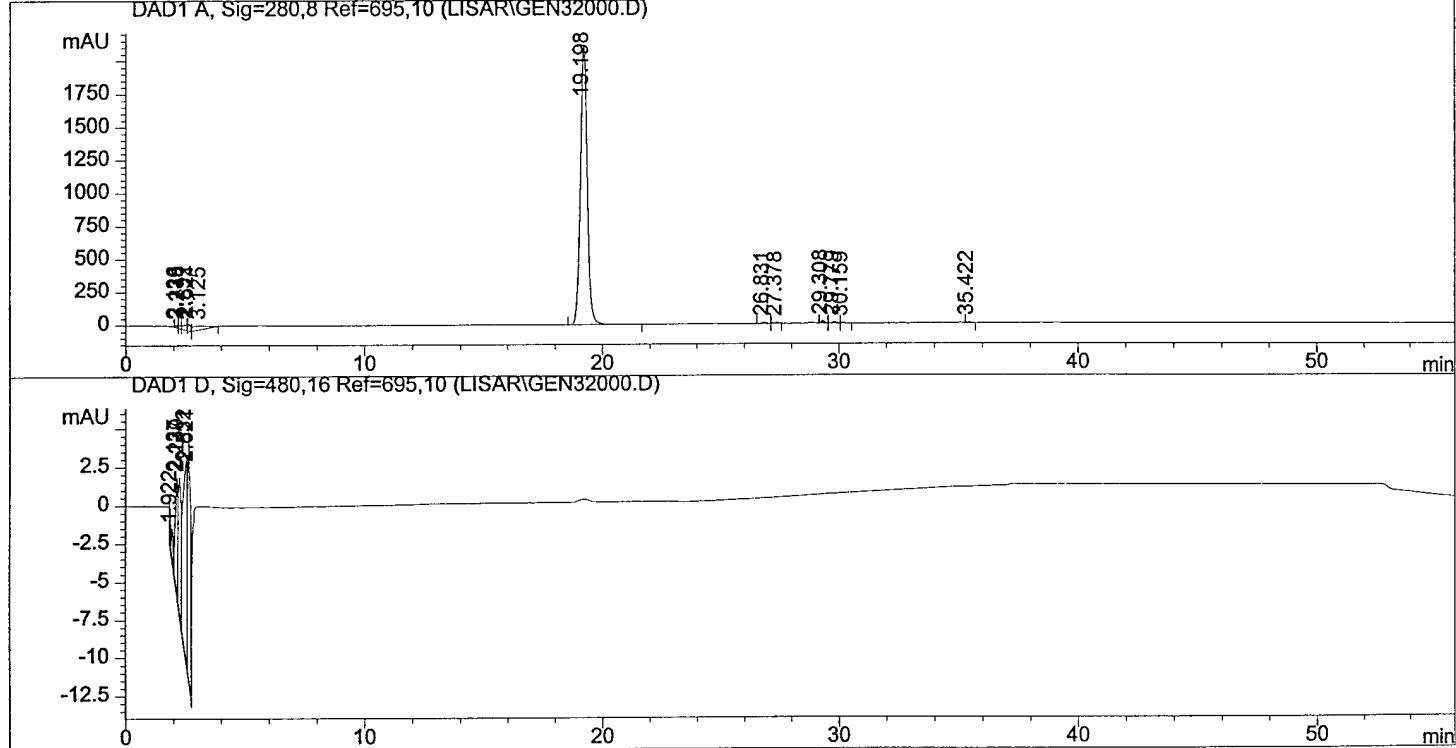
=====
   
 Injection Date : 3/20/00 7:29:34 PM Seq. Line : 3
   
 Sample Name : Sanpah Vial : 3
   
 Acq. Operator : Kenny Inj : 1
   
 Inj Volume : 50  $\mu$ l
   
 Different Inj Volume from Sequence ! Actual Inj Volume : 20  $\mu$ l
   
 Sequence File : C:\HPCHEM\1\SEQUENCE\EGF32000.S
   
 Method : C:\HPCHEM\1\METHODS\LISETGF2.M
   
 Last changed : 3/20/00 5:08:16 PM by Kenny
   
 EGF samples with Lichrospher 100 column. C: H<sub>2</sub>O, 0.1% TFA D: 80% ACN 20% H<sub>2</sub>O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43.5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



=====
   
**Figure 1D -** Figure 1D shows the reverse-phase HPLC pattern of a Sulfo-SANPAH control.

Genistein from 3/13/00. 1mg Gen/30ul DMSO.  
 5 ul Sanpah + 250 uL MeOH in HPLC vial.  
 LiChrospher 100 column (RP-18, 5um).

```
=====
Injection Date : 3/20/00 6:22:27 PM           Seq. Line : 2
Sample Name    : Genistein                 Vial   : 2
Acq. Operator   : Kenny                   Inj    : 1
                                         Inj Volume : 50 μl
Different Inj Volume from Sequence !     Actual Inj Volume : 20 μl
Sequence File  : C:\HPCHEM\1\SEQUENCE\EGF32000.S
Method         : C:\HPCHEM\1\METHODS\LISAEGF2.M
Last changed   : 3/20/00 5:08:16 PM by Kenny
EGF samples with Lichrospher 100 column. C: H20, 0.1% TFA D: 80% ACN 20%
H2O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43% D;
t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.
```

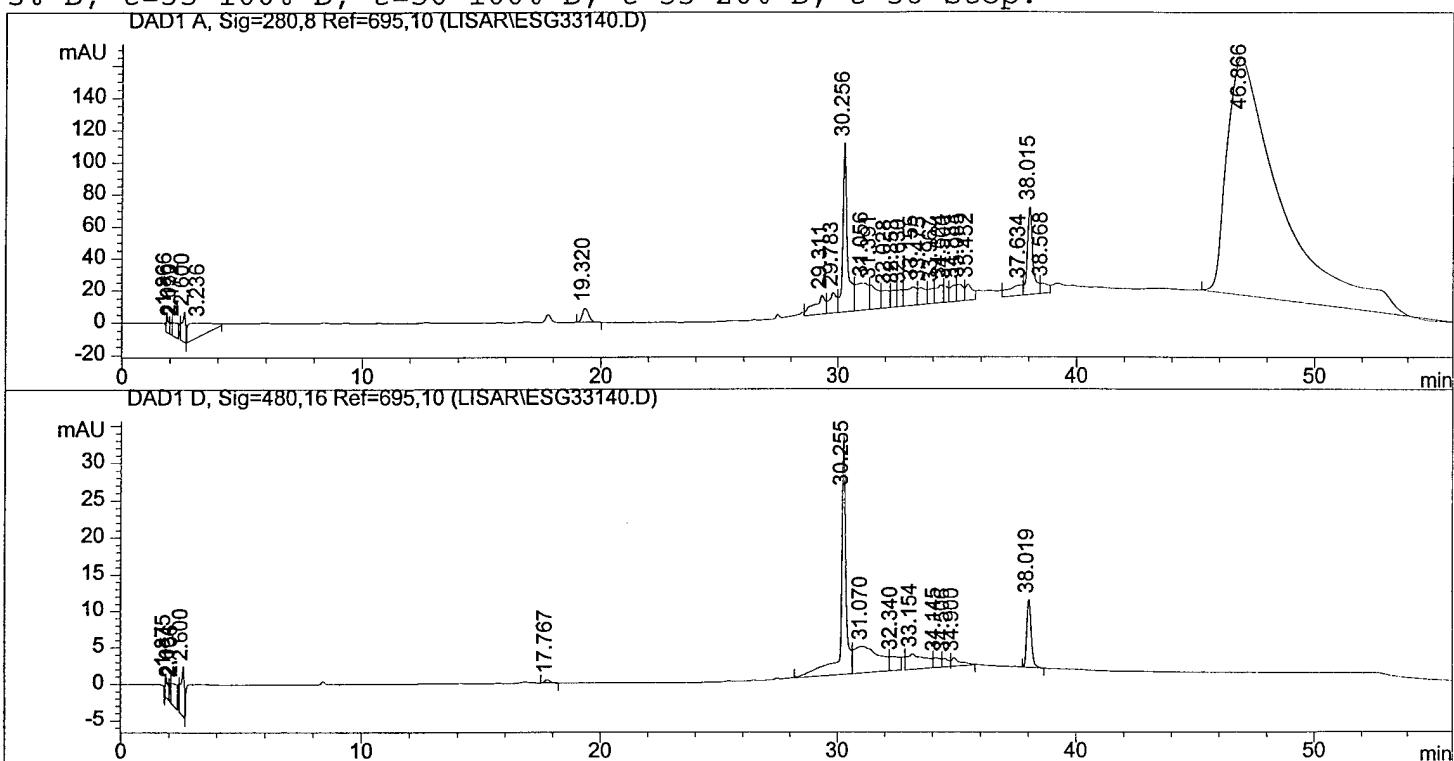


**Figure 1E** - Figure 1E shows the HPLC pattern for Genistein.

EGF/SAN/GEN (1:10, 1:10) 1 HR LWUV.  
 Diluted to 1 mg/ml in PBS. Made 3/6/00.  
 Run previously on HPLC. Stored at -20.

```
=====
Injection Date : 3/15/00 3:03:34 AM           Seq. Line : 12
Sample Name    : EGF/SAN/GEN                 Vial    : 9
Acq. Operator   : Deb                      Inj     : 1
                                                Inj Volume : 50 μl
Different Inj Volume from Sequence !      Actual Inj Volume : 25 μl
Sequence File  : C:\HPCHEM\1\SEQUENCE\EGF31400.S
Method         : C:\HPCHEM\1\METHODS\LISAEGF2.M
Last changed   : 3/15/00 3:02:14 AM by Deb
                           (modified after loading)
```

EGF samples with Lichrospher 100 column. C: H<sub>2</sub>O, 0.1% TFA D: 80% ACN 20% H<sub>2</sub>O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43.5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



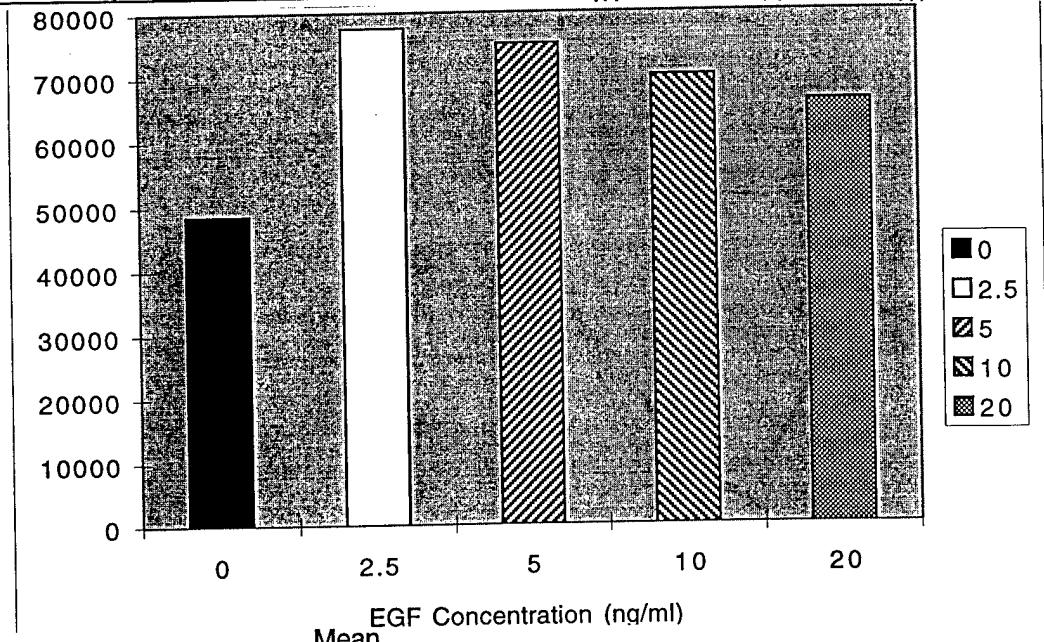
**Figure 1F** - Figure 1F shows a reverse-phase HPLC pattern of EGF-SANPAH - Gen made by photolyzing the EGF-SANPAH in the presence of a 10:1 molar excess of Genistein. This preparation contains much less free Genistein.

**Figures 2A and 2B** - Figure 2A shows the effects of various concentrations of EGF on the growth of CRL-1634 cells incubated for five days. The control cells received no EGF. Figure 2B indicates that incubation of EGF in the presence of various concentrations of DMSO results in inhibition of the growth of CRL-1634 cells. Excess DMSO was removed by passage of the EGF through a "desalting" column prior to incubation with the cells.

EGF Concentration (ng/ml)	Mean cells/ml	Std deviation	Std error	Fold increase or decrease (-)	Percentage Increase
				to control	
0	48500	4725.82	2362.91		
2.5	77500	14730.92	7365.46	1.6	37.4
5	75000	10893.42	5446.71	1.5	35.3
10	70000	23664.32	11832.16	1.4	30.7
20	66000	9797.96	4898.98	1.4	26.5

59.8% of o EGF  
54.6% " " "  
44.3% " " "  
36.1% " " "

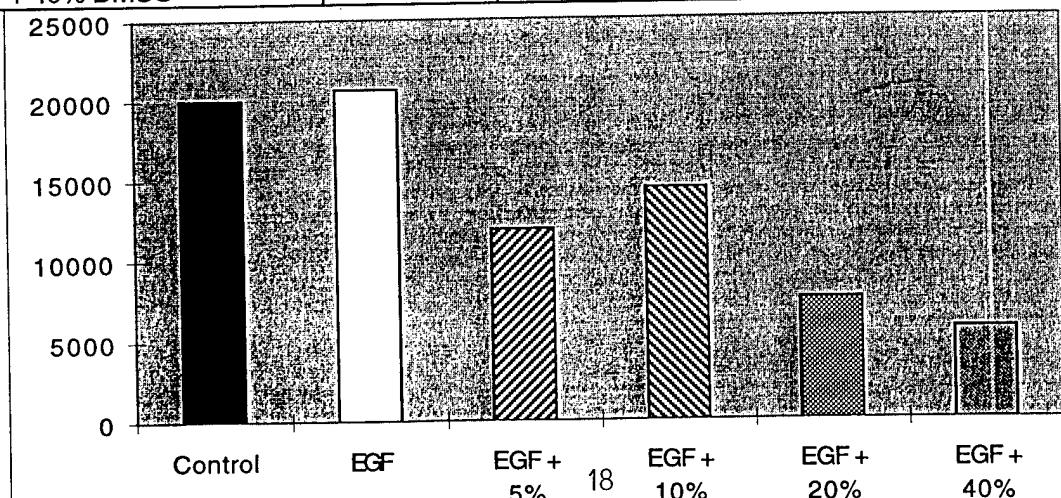
Figure 2A



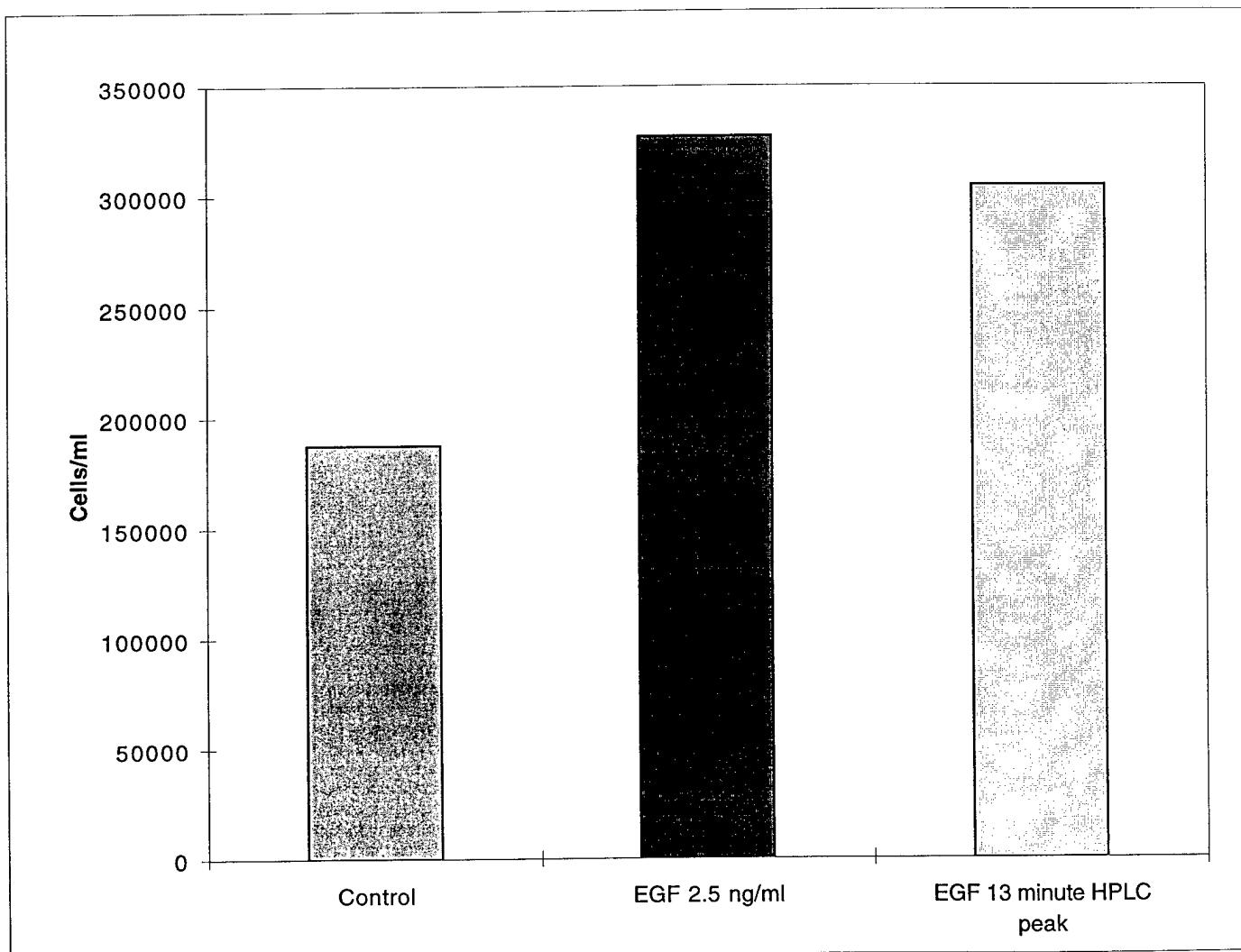
Compound Tested

Compound Tested	Mean cells/ml	Std deviation	Std error
Control	20000	8416	4208
EGF	20625	4270	2135
EGF + 5% DMSO	11875	4270	2135
EGF + 10% DMSO	14375	5154	2577
EGF + 20% DMSO	7500	3536	1768
EGF + 40% DMSO	5625	4732	2366

Figure 2B

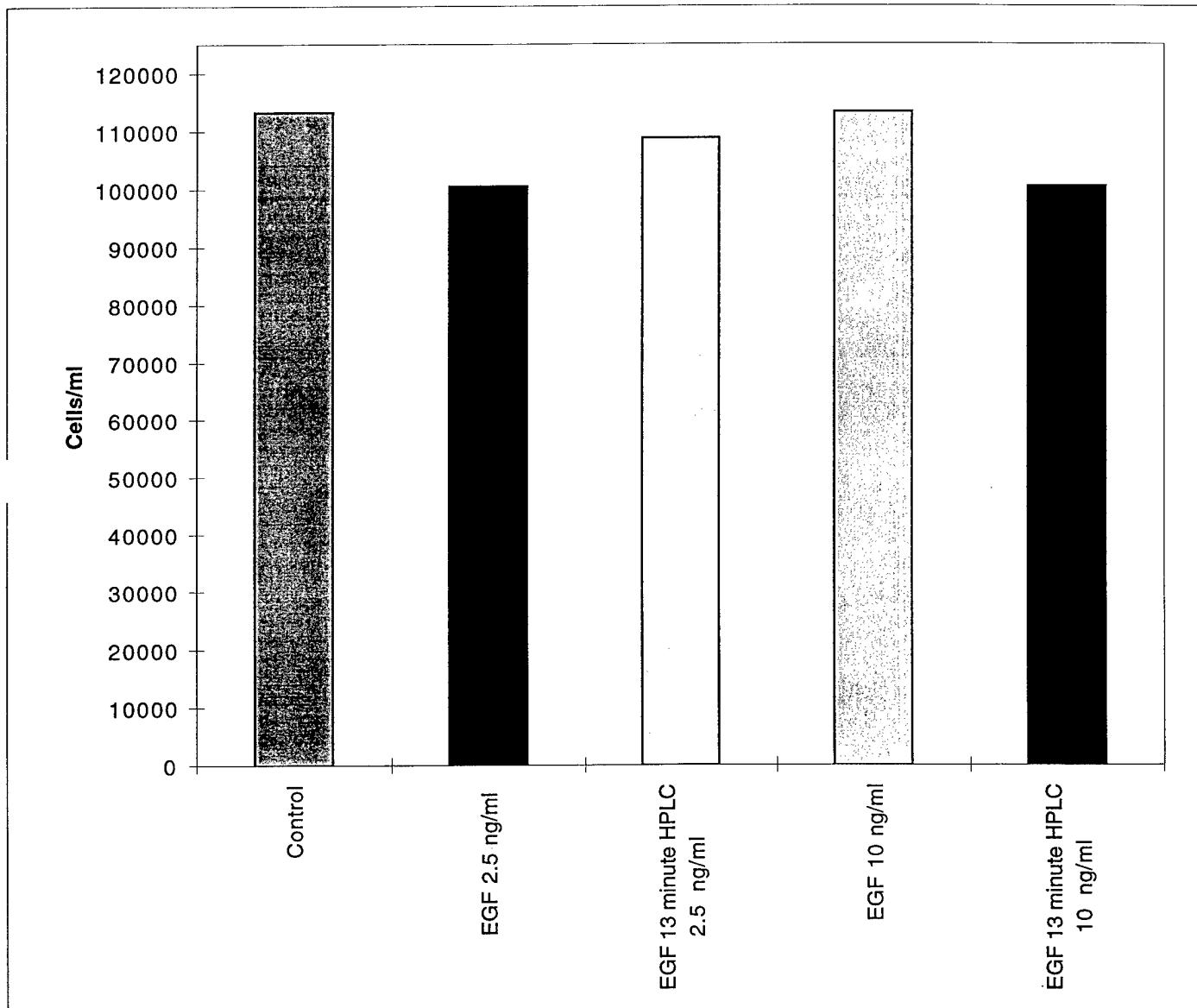


	Mean cells/ml	Std deviation	Std error
Control	187500	17916	8958
EGF 2.5 ng/ml 3/28/00	327000	64946	32473
EGF 13 minute HPLC peak	304500	15199	7599
EGF 2.5 ng/ml 3/7/00	298500	55344	27672
EGF 2.5 ng/ml 3/13/00	362250	28500	14250
EGF 2.5 ng/ml 3/20/00	304500	12610	6305



**Figure 3** - Figure 3 shows that EGF is still able to stimulate CRL-1634 cells after passage through a reverse-phase column in the presence of acetonitrile. The acetonitrile is evaporated under nitrogen and the protein concentration determined by the BCA assay.

	Mean cells/ml	Std deviation	Std error
Control	113250	18392	9196
EGF 2.5 ng/ml 3/28/00	100500	19672	9836
EGF 13 minute HPLC peak 2.5 ng/ml	108750	16132	8066
EGF 10 ng/ml 3/28/00	113250	14773	7387
EGF 13 minute HPLC peak 10 ng/ml	100500	14387	7194



MDAMB231 cells were incubated for 5 days prior to counting.

**Figure 4 - Results of an EGF Growth Assay showing MDA-MB-231 cells incubated in the presence of two concentrations of Stock EGF and HPLC-purified EGF.**

Compound Tested	Mean cells/ml	Std deviation	Std error
Control	18750	6652.07	3326.03
EGF 2.5ng/ml	39750	8958.24	4479.12
EGF/SAN 2.5 ng/ml	45000	19748.42	9874.21
EGF/GEN 2.5 ng/ml	42000	11224.97	5612.49

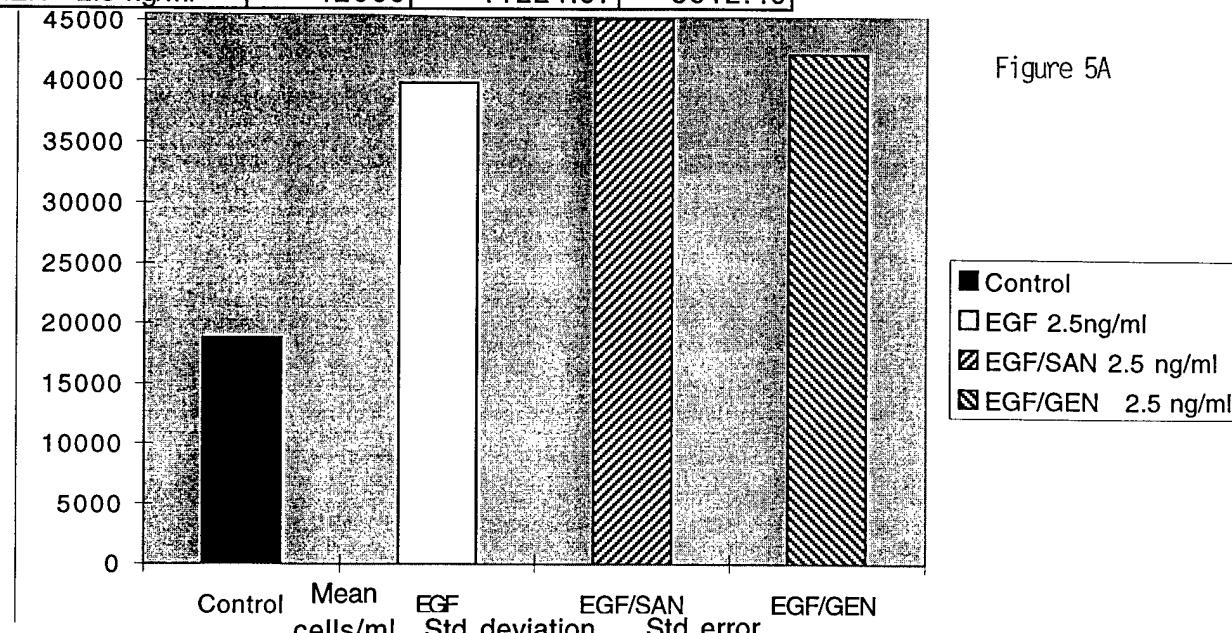
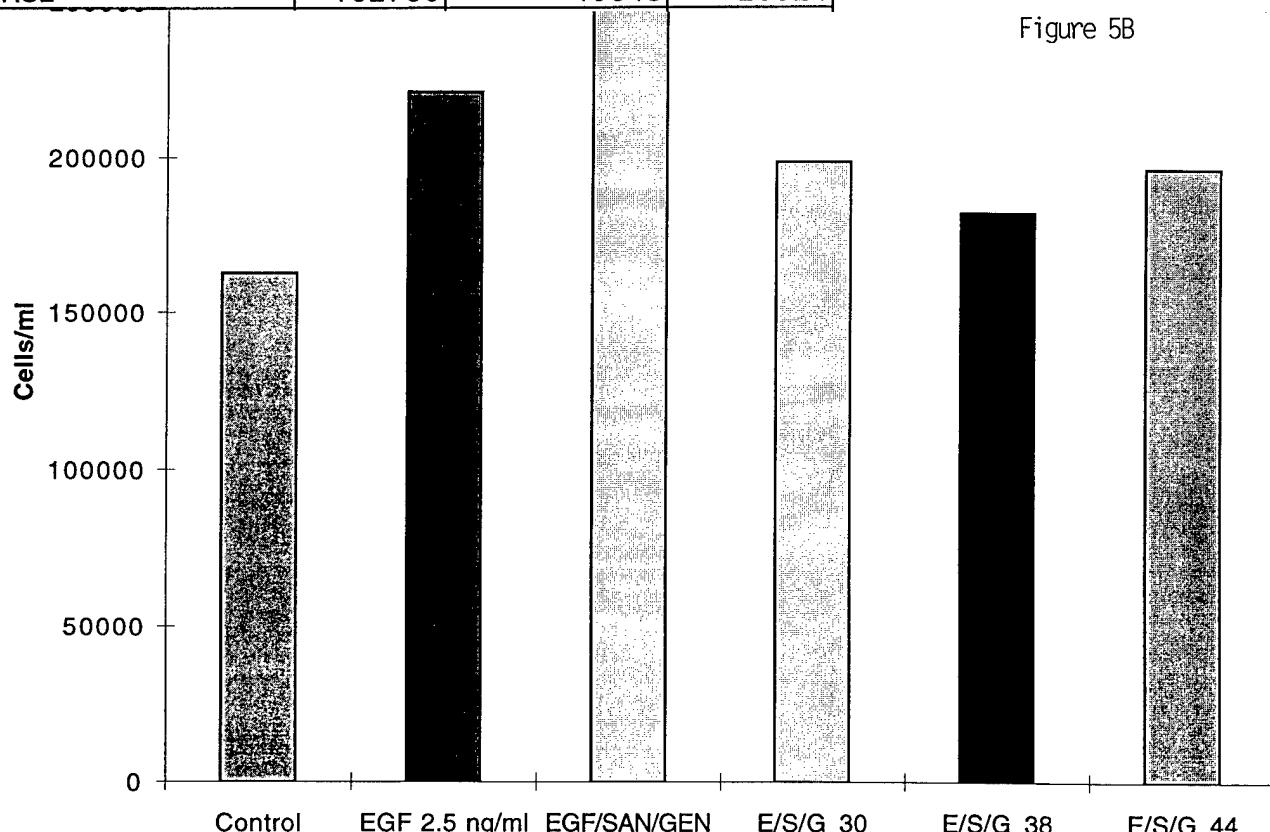


Figure 5A

	Mean cells/ml	EGF Std deviation	EGF/SAN Std error	EGF/GEN Std error
EGF 2.5 ng/ml	221250	14361	7181	
EGF/SAN/GEN (1:10,1:20)	247500	24799	12400	
E/S/G 30 minute HPLC	198750	12093	6047	
E/S/G 38 minute HPLC	182250	28814	14407	
E/S/G 44 minute HPLC	196500	13528	6764	
CONTROL	162750	40648	20324	

Figure 5B

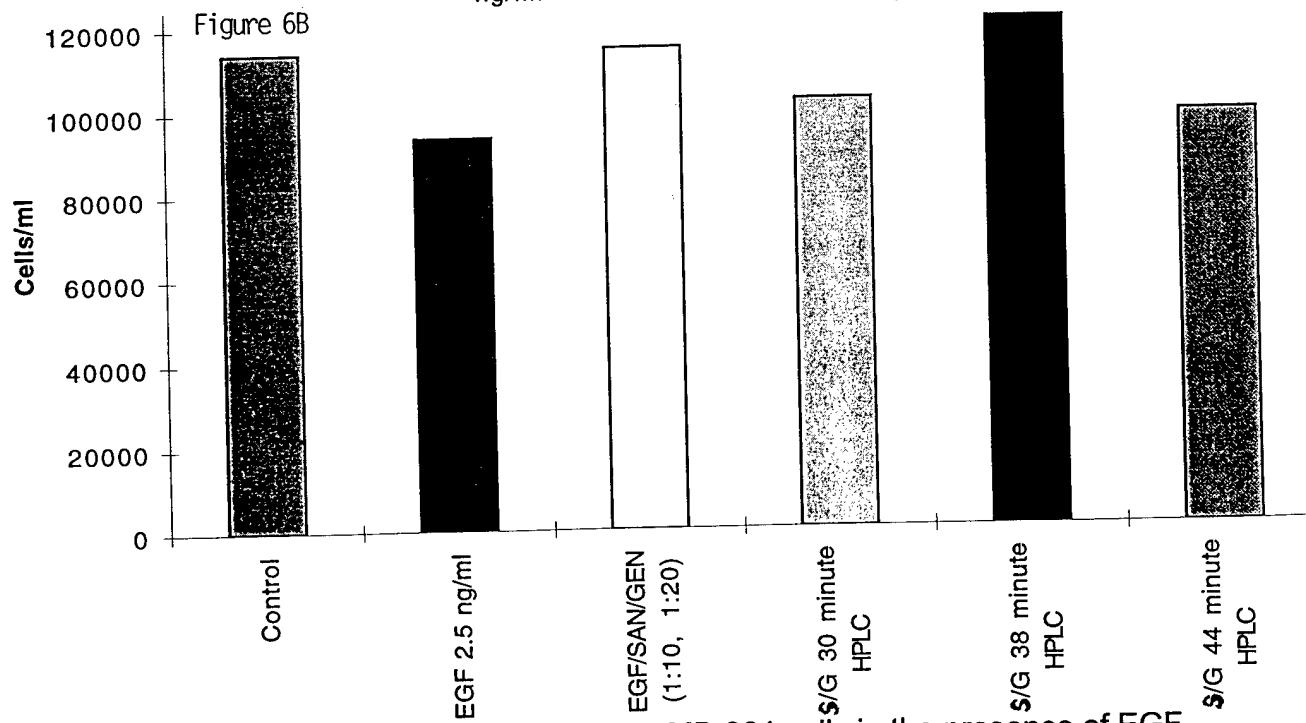
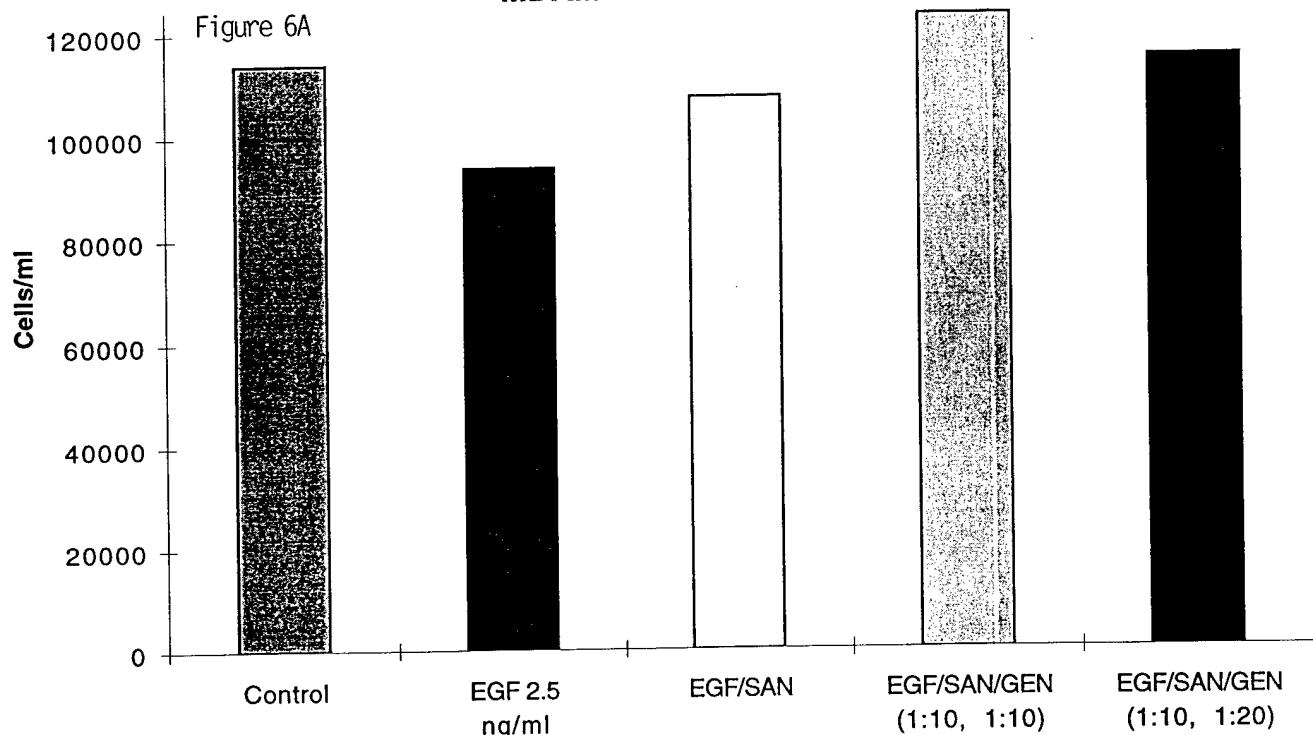


**Figures 5A and 5B -** Figures 5A and 5B show the growth of CRL-1634 cells

incubated in the presence of EGF-SANPAH and EGF-SAN-Gen, the parent conjugates as well as the fractions isolated from the reverse-phase HPLC.

Compound Tested	Mean cells/ml	Std deviation	Std error
Control	114000	20928	10464
EGF/SAN	107250	22096	11048
EGF/SAN/GEN (1:10, 1:10)	123000	22181	11091
EGF/SAN/GEN (1:10, 1:20)	114750	4500	2250
EGF 2.5 ng/ml	93750	8958	4479
E/S/G 30 minute HPLC	102000	10392	5196
E/S/G 38 minute HPLC	120750	9912	4956
E/S/G 44 minute HPLC	98250	13937	6969
EGF 10 ng/ml	126750	26986	13493

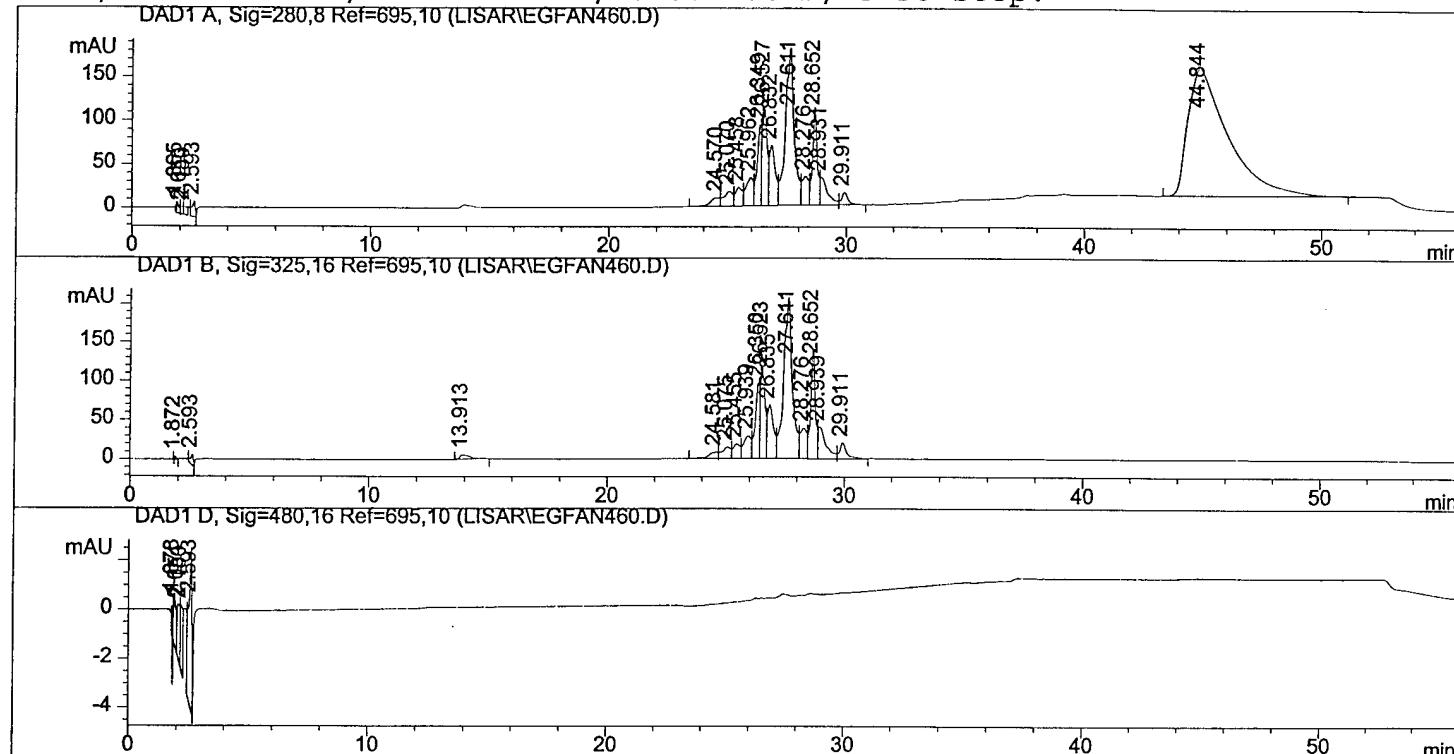
### MDAMB-231 CELLS



**Figure 6A and 6B - Incubation of MDA-MB-231 cells in the presence of EGF-SANPAH and EGF-SAN-Gen, the parent compounds as well as the fractions isolated from the reverse-phase HPLC.**

EGF/ANB-NOS (1:10) 4/6/00  
 Lichrospher column (RP-18, 5um) Flow = 1 ml/min.

```
=====
Injection Date : 4/6/00 1:54:59 PM          Seq. Line : 4
Sample Name    : EGF/ANB-NOS            Vial : 4
Acq. Operator   : Joe                  Inj : 1
                                                Inj Volume : 100 µl
Different Inj Volume from Sequence !      Actual Inj Volume : 25 µl
Sequence File  : C:\HPCHEM\1\SEQUENCE\EGF4600.S
Method         : C:\HPCHEM\1\METHODS\LISAEGF2.M
Last changed   : 4/6/00 10:26:29 AM by Joe
EGF samples with Lichrospher 100 column. C: H2O, 0.1% TFA D: 80% ACN 20%
H2O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43.
5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.
```



**Figure 7A** - Figure 7A shows an HPLC pattern of EGF modified with a 10:1 molar ratio of the ANB-NOS crosslinker. Unmodified EGF would elute at 13 - 14 min, ANB-NOS at 23 - 24 min. UV scans are shown for the peaks at 26.527, 27.611, and 28.652 min. The peaks show both the 280 nm and 325 nm

Int of window 39: UV Apex spectrum of Peak 26.527 of EGFAN460.D

UV Apex spectrum of Peak 26.527 of EGFAN460.D

\*DAD1, 26.527(200 mAU, -) Ref=26.427 & 26.627 of EGFAN460.D

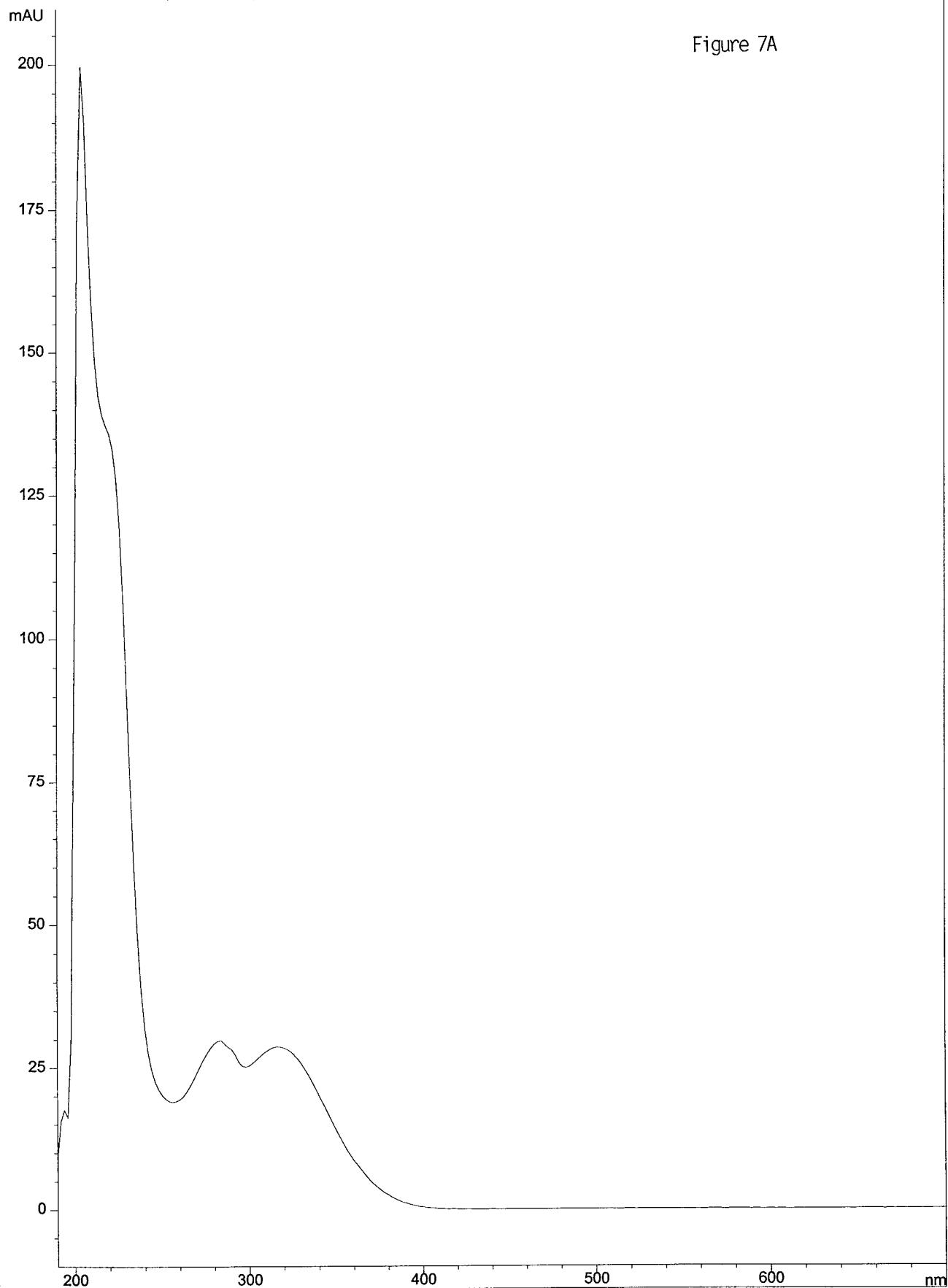


Figure 7A

UV Apex spectrum of Peak 27.611 of EGFAN460.D

\*DAD1, 27.614 (427 mAU, -) Ref=27.440 & 27.780 of EGFAN460.D

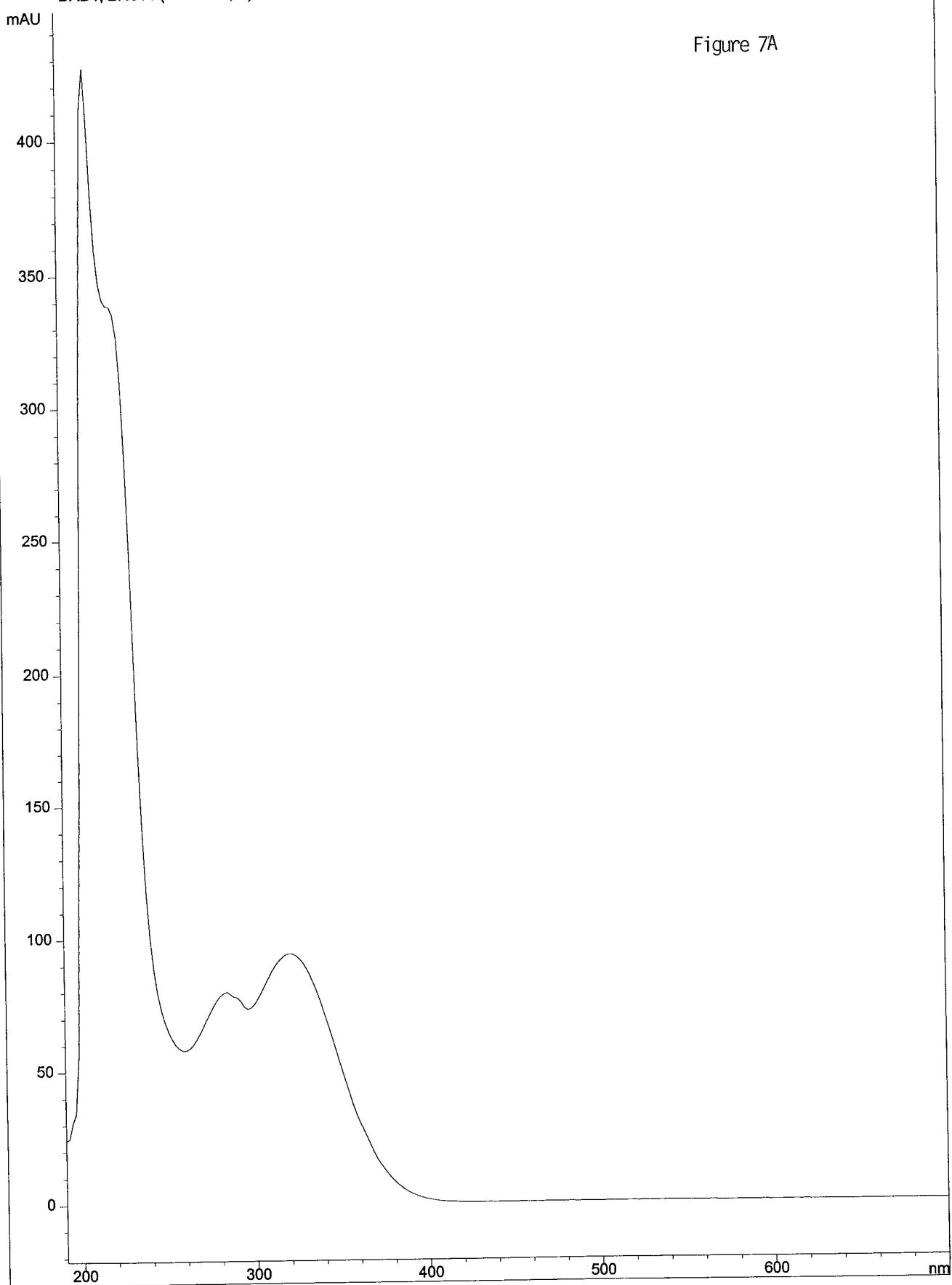


Figure 7A

UV Apex spectrum of Peak 28.652 of EGFAN460.D

\*DAD1, 28.654 (392 mAU, -) Ref=28.554 & 28.747 of EGFAN460.D

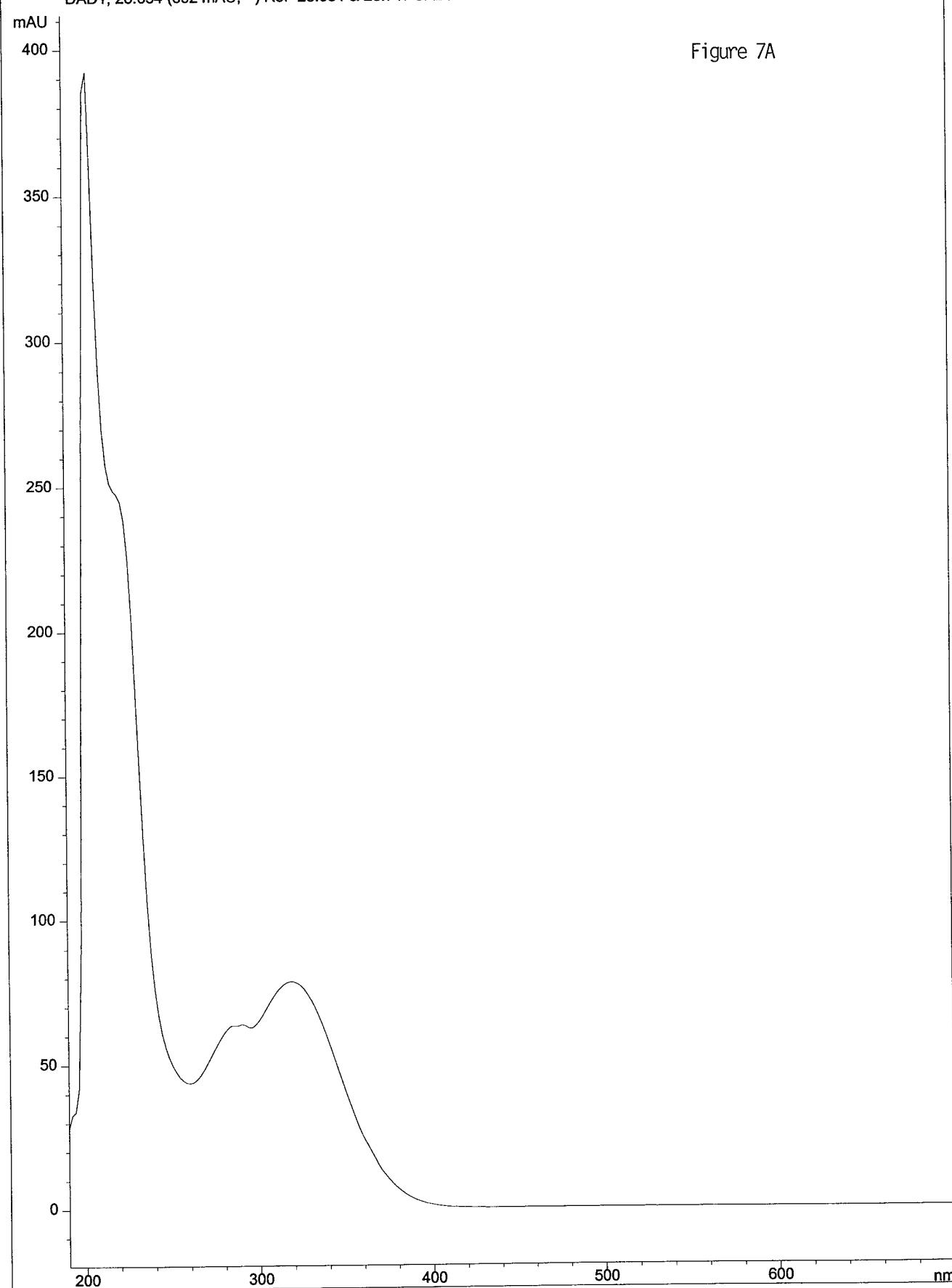


Figure 7A

EGF/ANB/GEN (1:10, 1:20) 1 HRLWUV 4/6/00  
 Lichrospher column (RP-18, 5 um) Flow = 1 ml/min

=====
   
 Injection Date : 4/6/00 4:09:24 PM Seq. Line : 6
   
 Sample Name : E/A/G 1:10, 1:20 Vial : 6
   
 Acq. Operator : Joe Inj : 1
   
 =====

Inj Volume : 100  $\mu$ l

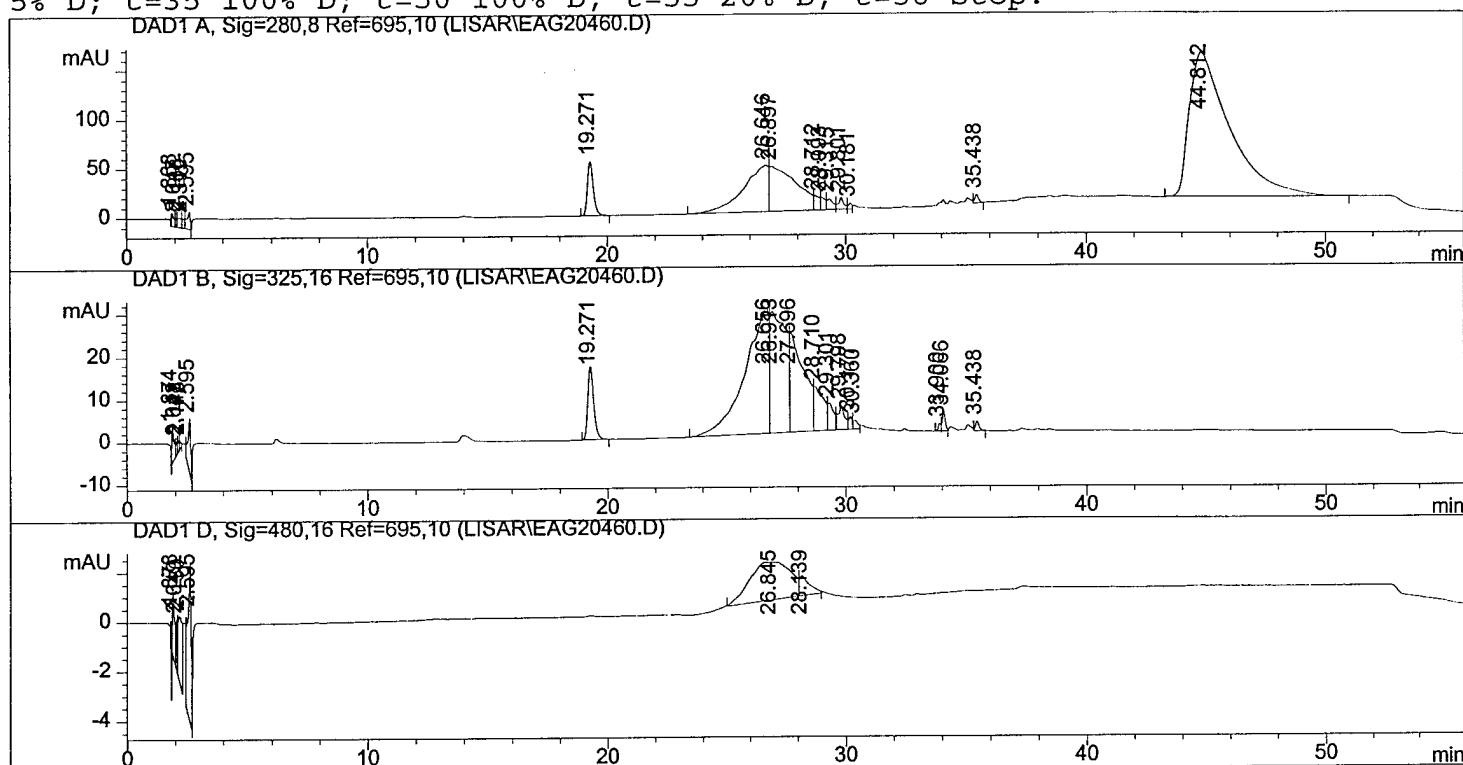
Different Inj Volume from Sequence ! Actual Inj Volume : 25  $\mu$ l

Sequence File : C:\HPCHEM\1\SEQUENCE\EGF4600.S

Method : C:\HPCHEM\1\METHODS\LISAEGF2.M

Last changed : 4/6/00 10:26:29 AM by Joe

EGF samples with Lichrospher 100 column. C: H2O, 0.1% TFA D: 80% ACN 20% H2O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43. 5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



**Figure 7B** - Figure 7B shows the HPLC pattern of the EGF-ANB-NOS-Gen conjugate photolyzed in the presence of a 20:1 molar excess of Genistein. Unreacted Genistein elutes at 19 - 20 min. A UV scan of the broad peak at 26.646 min is included.

rt of window 39: UV Apex spectrum of Peak 26.646 of EAG20460.D

UV Apex spectrum of Peak 26.646 of EAG20460.D

\*DAD1, 26.644 (53.3 mAU, -) Ref=26.197 of EAG20460.D

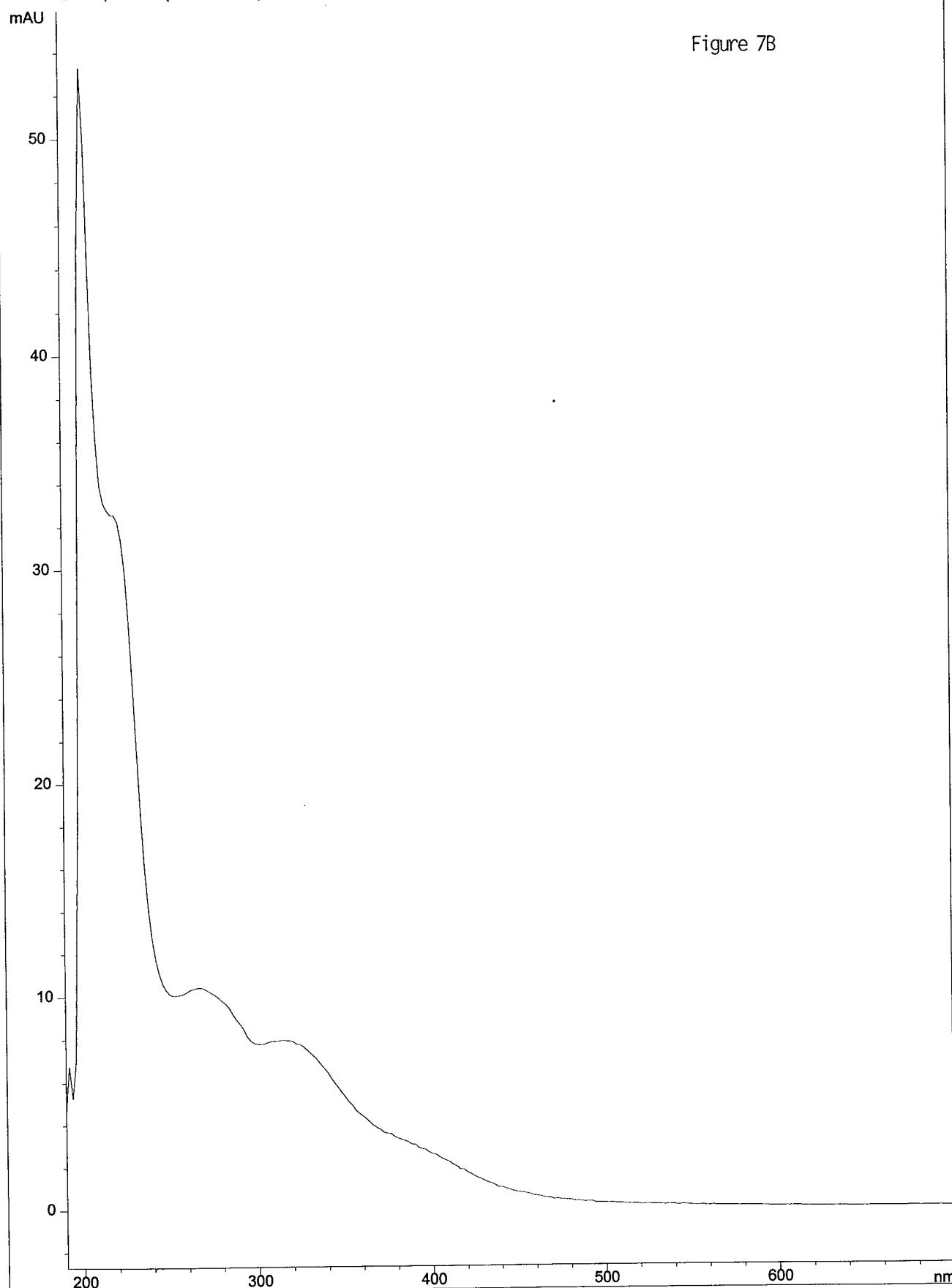
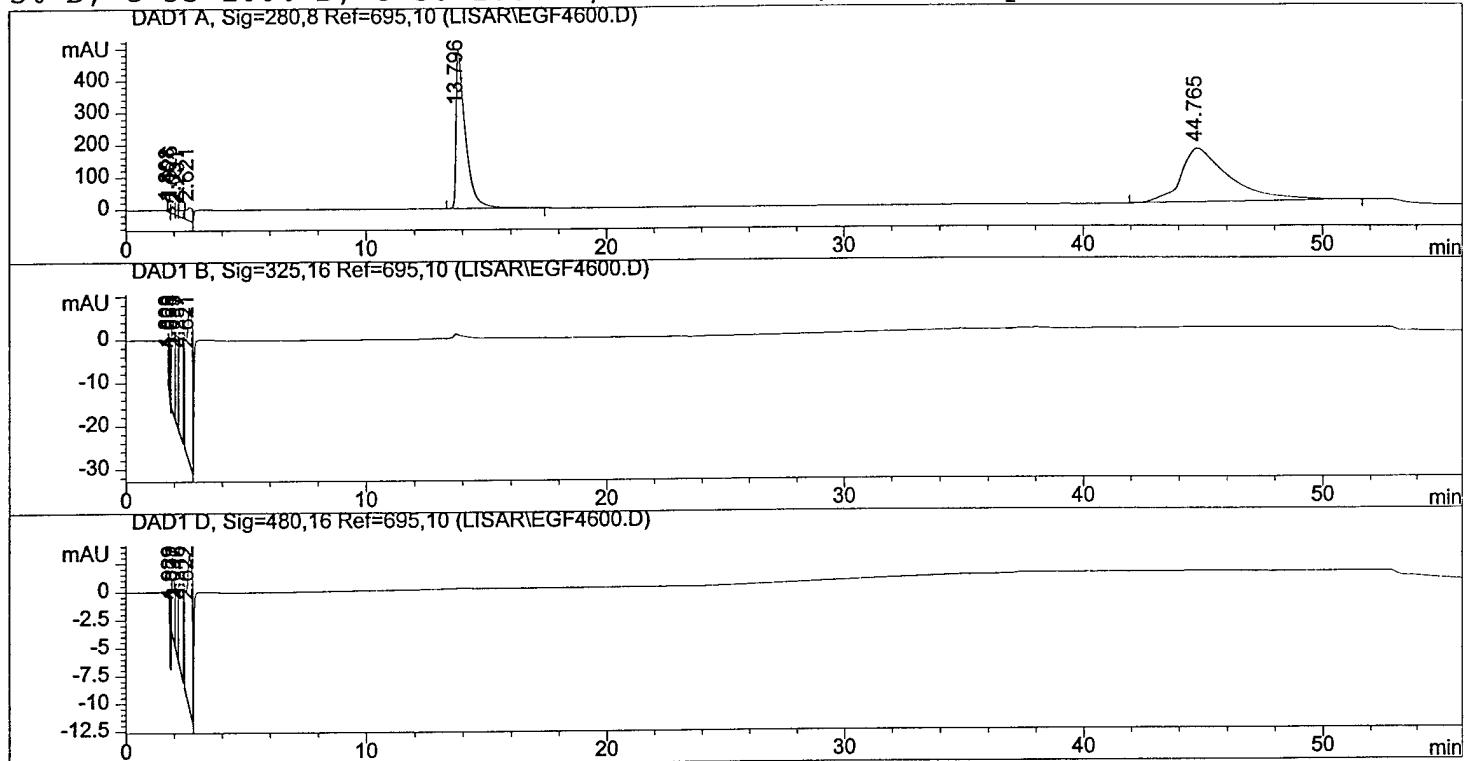


Figure 7B

EGF 1 mg/ml in PBS 4/6/00  
 Lichrospher column (RP-18, 5um) Flow = 1 ml/min

=====
   
 Injection Date : 4/6/00 12:47:28 PM Seq. Line : 3
   
 Sample Name : EGF Vial : 3
   
 Acq. Operator : Joe Inj : 1
   
 Inj Volume : 100  $\mu$ l
   
 Sequence File : C:\HPCHEM\1\SEQUENCE\EGF4600.S
   
 Method : C:\HPCHEM\1\METHODS\LISAEGF2.M
   
 Last changed : 4/6/00 10:26:29 AM by Joe
   
 EGF samples with Lichrospher 100 column. C: H<sub>2</sub>O, 0.1% TFA D: 80% ACN 20%
   
 H<sub>2</sub>O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43% D;
   
 t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



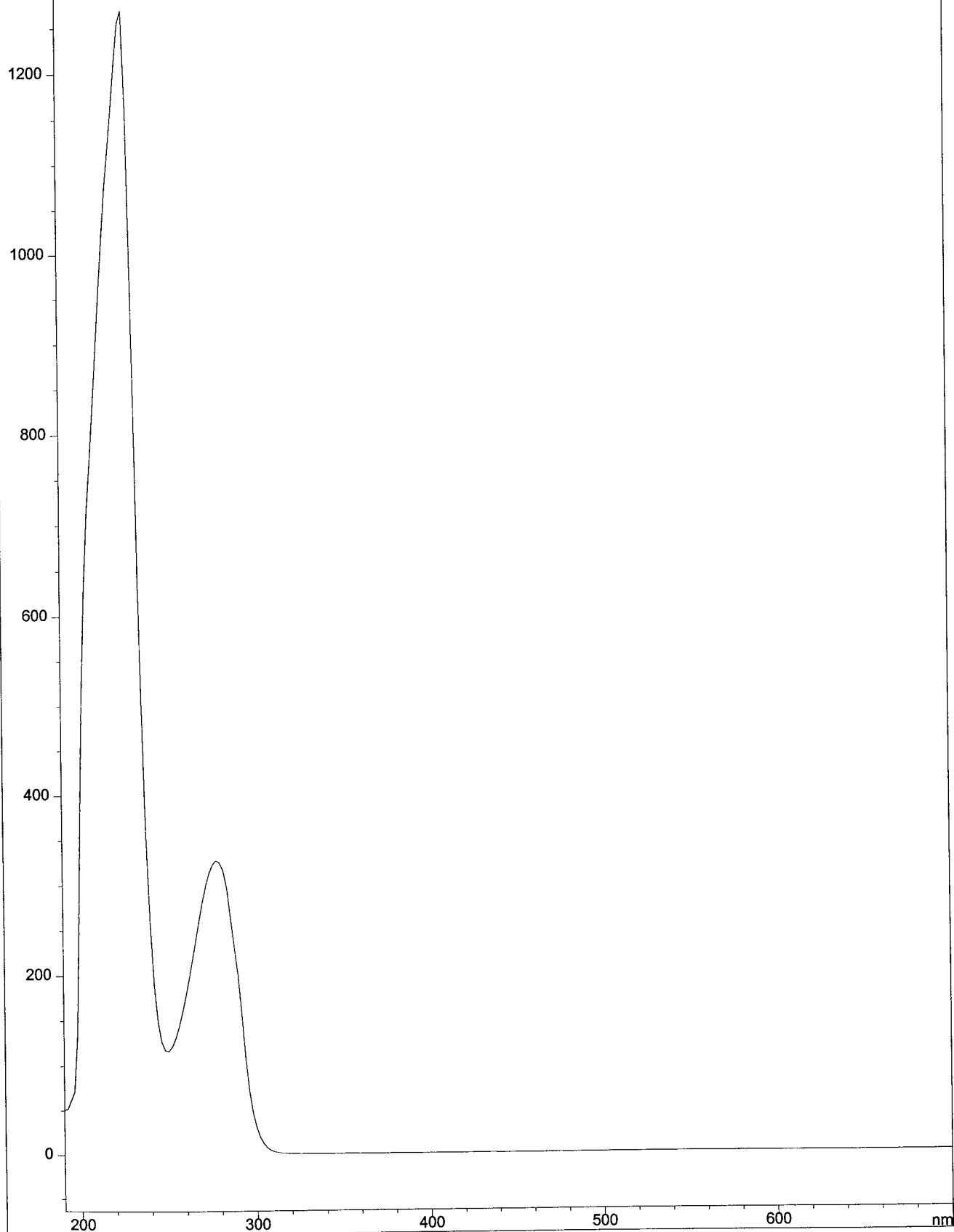
**Figure 7C** - Figure 7C shows the HPLC pattern and UV scan of EGF.

UV Apex spectrum of Peak 13.796 of EGF4600.D

\*DAD1, 13.797 (1269 mAU, - ) Ref=13.610 & 13.977 of EGF4600.D

mAU

Figure 7C



UV Apex spectrum of Peak 44.765 of EGF4600.D  
\*DAD1, 44.764 (777 mAU, -) Ref=43.797 & 45.730 of EGF4600.D

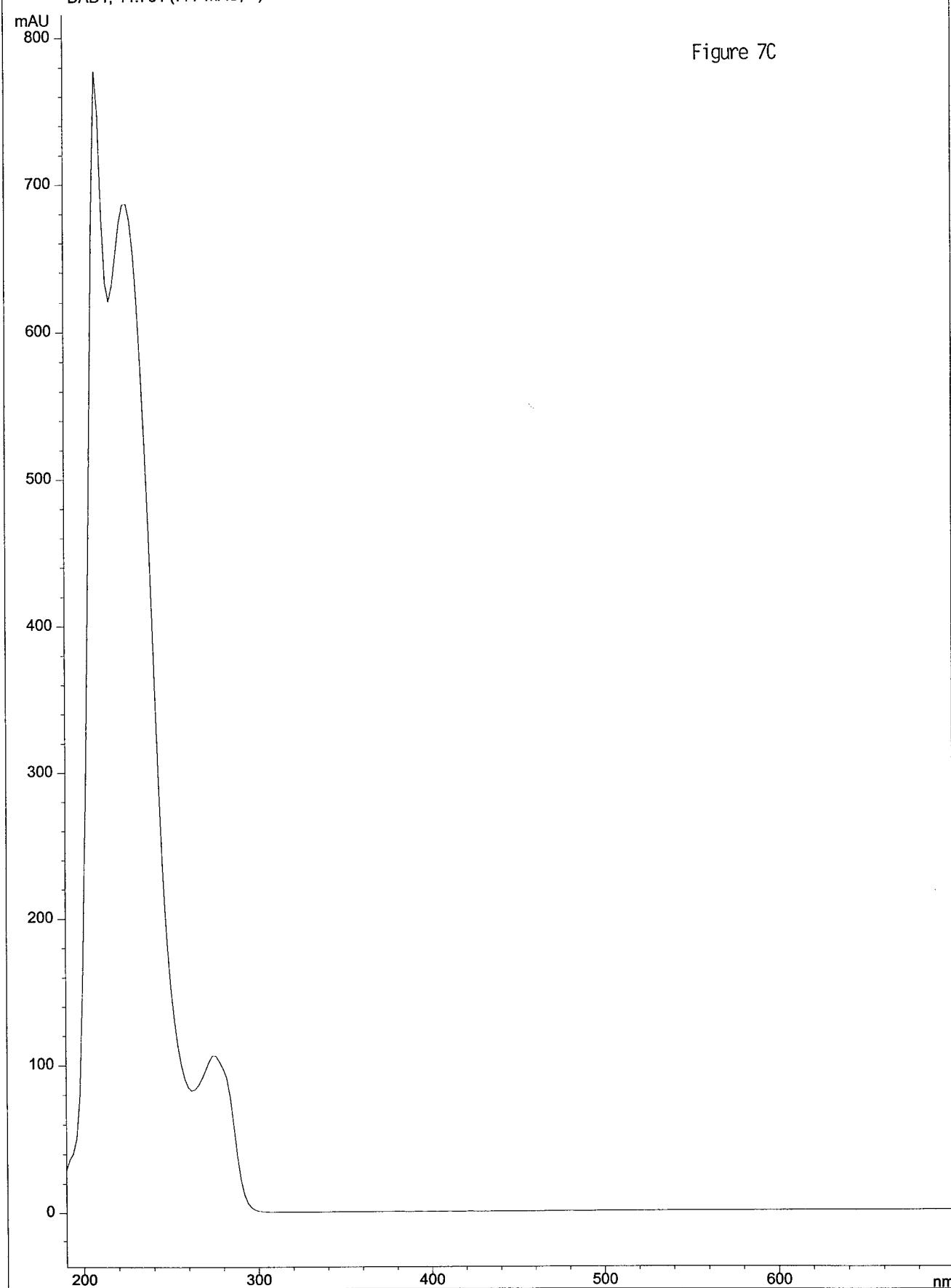
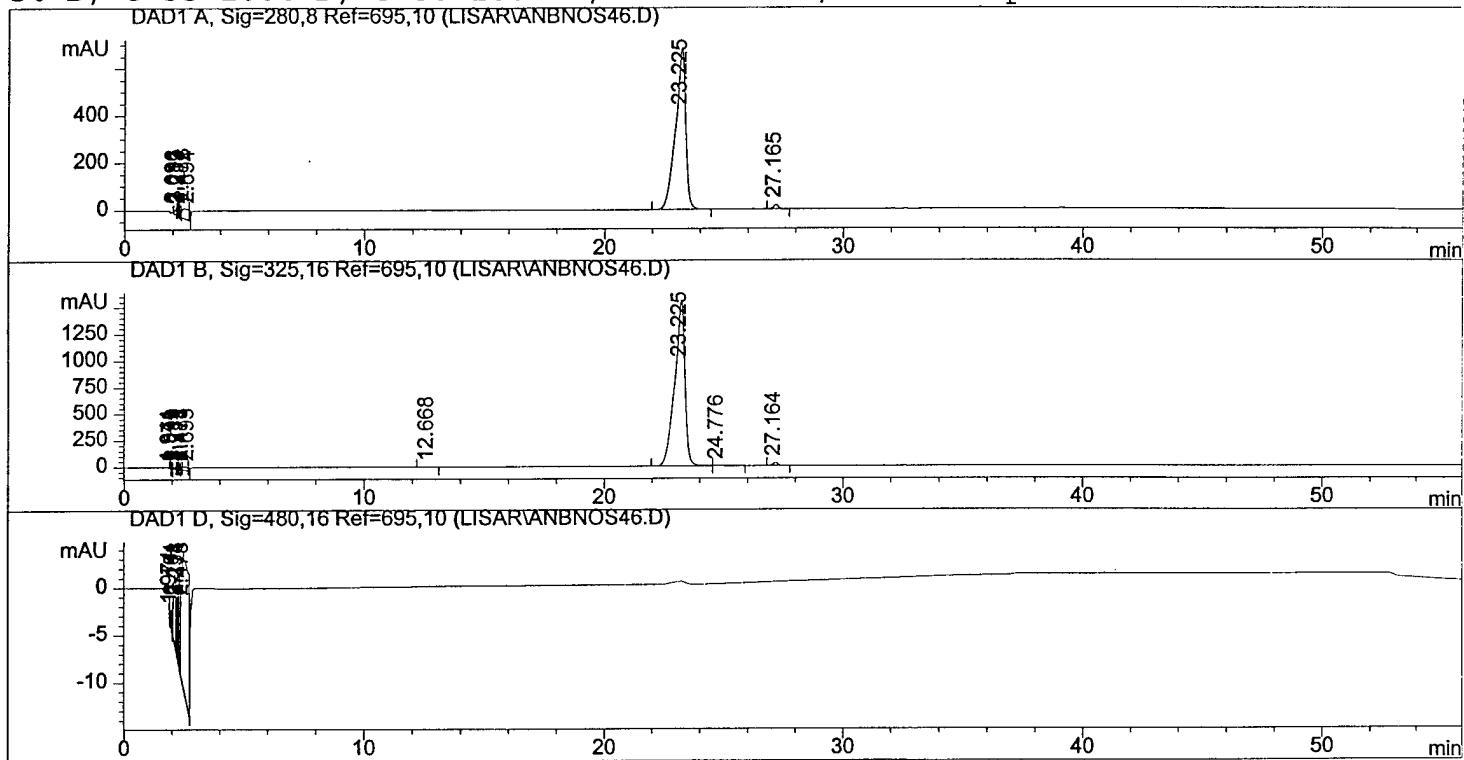


Figure 7C

ANB NOS 4/6/00 5 ul + 250 ul methanol.  
 LiChrospher 100 column (RP-18, 5um). Flow = 1 ml/min

Fatih

```
=====
Injection Date : 4/6/00 11:39:58 AM           Seq. Line : 2
Sample Name   : ANB NOS                   Vial   : 2
Acq. Operator  : Joe                      Inj    : 1
                                                Inj Volume : 100 μl
Different Inj Volume from Sequence !      Actual Inj Volume : 20 μl
Sequence File : C:\HPCHEM\1\SEQUENCE\EGF4600.S
Method        : C:\HPCHEM\1\METHODS\LISAEGF2.M
Last changed  : 4/6/00 10:26:29 AM by Joe
EGF samples with Lichrospher 100 column. C: H2O, 0.1% TFA D: 80% ACN 20%
H2O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43% D;
t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.
```



**Figure 7D** - Figure 7D shows the HPLC pattern and UV scan of an ANB-NOS standard.

UV Apex spectrum of Peak 23.225 of ANBNOS46.D

DAD1, 23.227 (929 mAU, -) Ref=23.020 & 23.434 of ANBNOS46.D

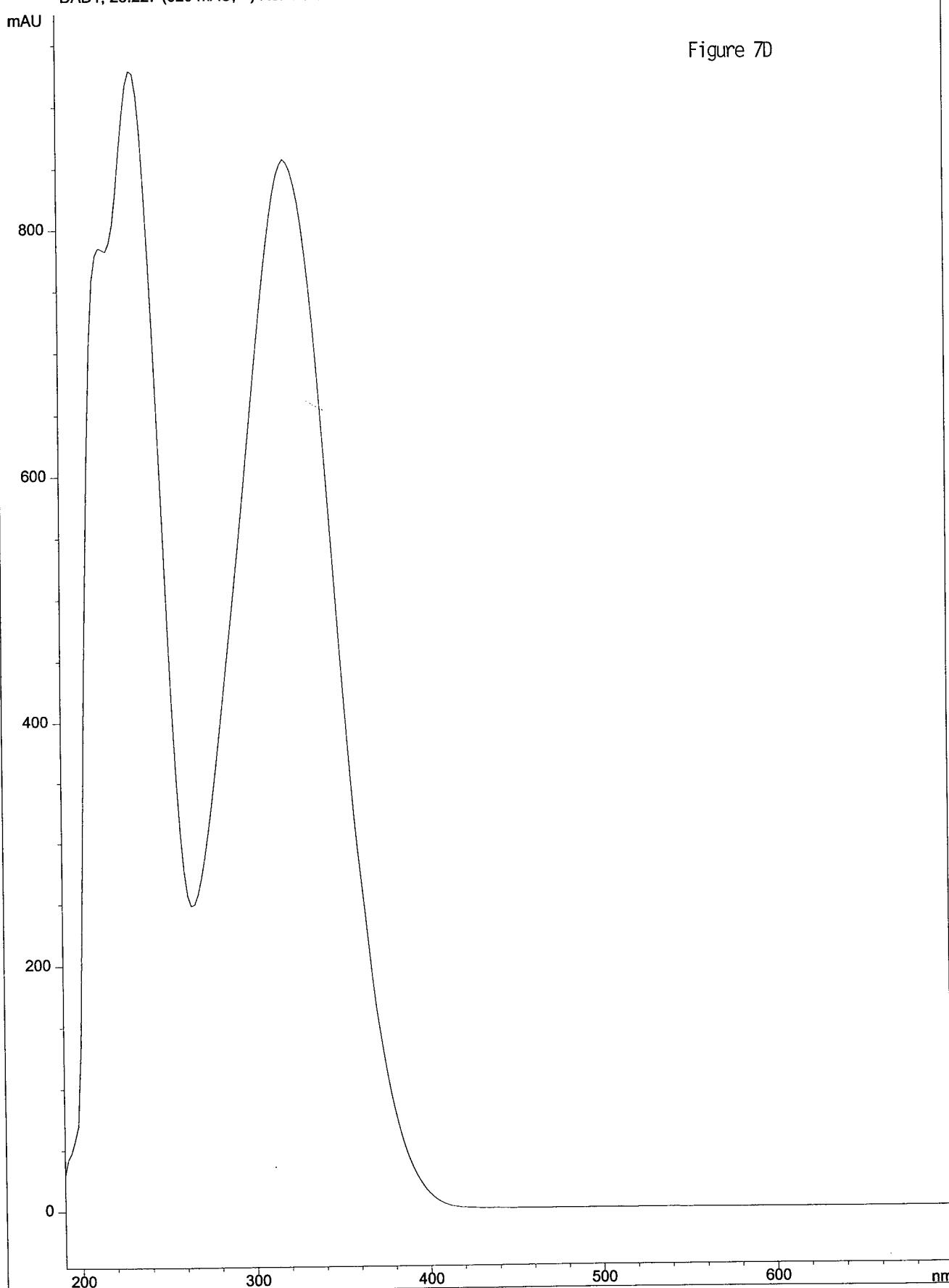


Figure 7D

Genistein 5/6/00 5ul + 250 ul methanol  
 Lichrospher column (RP-18, 5 um) Flow = 1 ml/min.

=====
   
 Injection Date : 4/6/00 10:32:49 AM Seq. Line : 1
   
 Sample Name : Genistein Vial : 1
   
 Acq. Operator : Joe Inj : 1

Inj Volume : 100  $\mu$ l

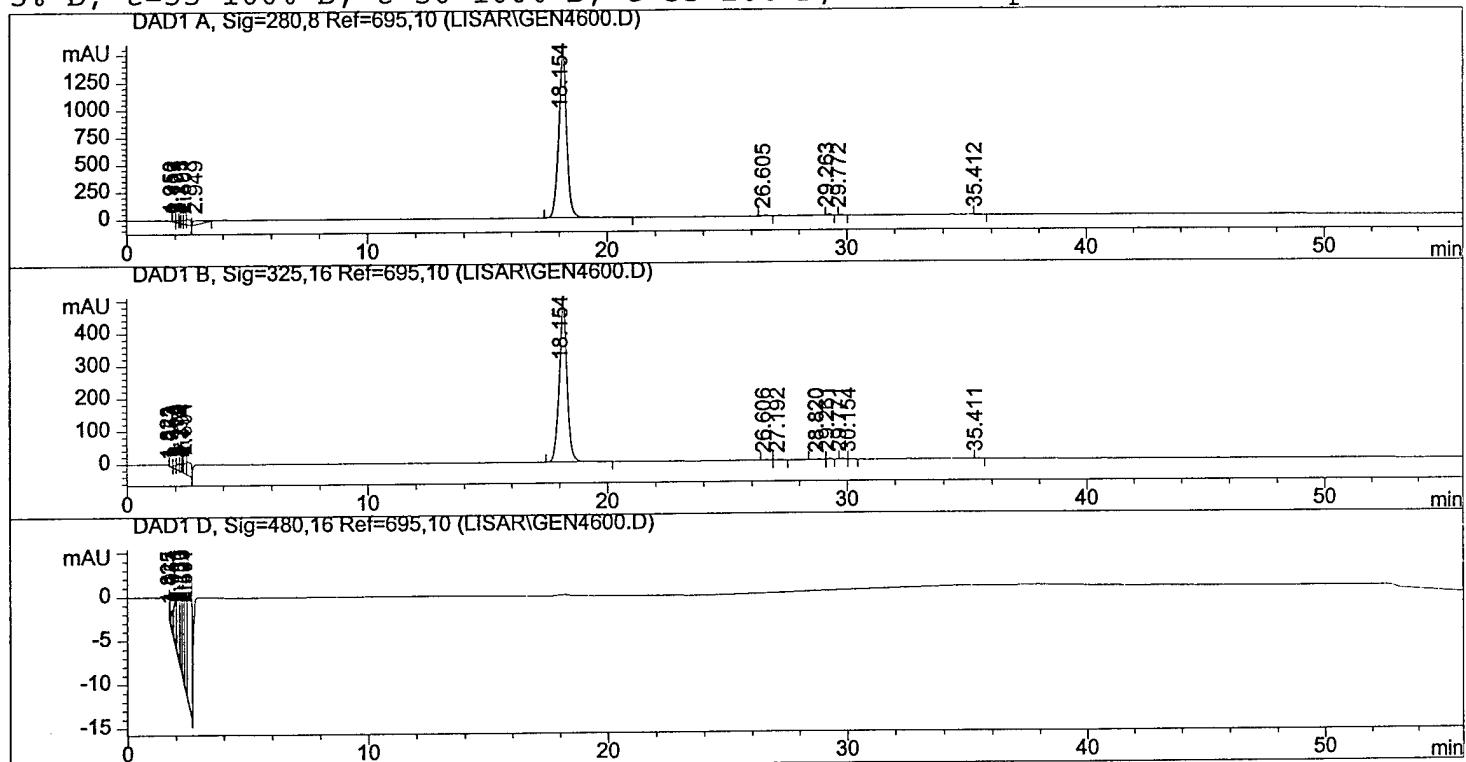
Different Inj Volume from Sequence ! Actual Inj Volume : 20  $\mu$ l

Sequence File : C:\HPCHEM\1\SEQUENCE\EGF4600.S

Method : C:\HPCHEM\1\METHODS\LISAEGF2.M

Last changed : 4/6/00 10:26:29 AM by Joe

EGF samples with Lichrospher 100 column. C: H2O, 0.1% TFA D: 80% ACN 20%  
 H2O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43%.  
 5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



**Figure 7E** - Figure 7E shows the HPLC pattern and UV scan of Genistein.

UV Apex spectrum of Peak 18.154 of GEN4600.D  
\*DAD1, 18.154 (1129 mAU, -) Ref=17.980 & 18.327 of GEN4600.D

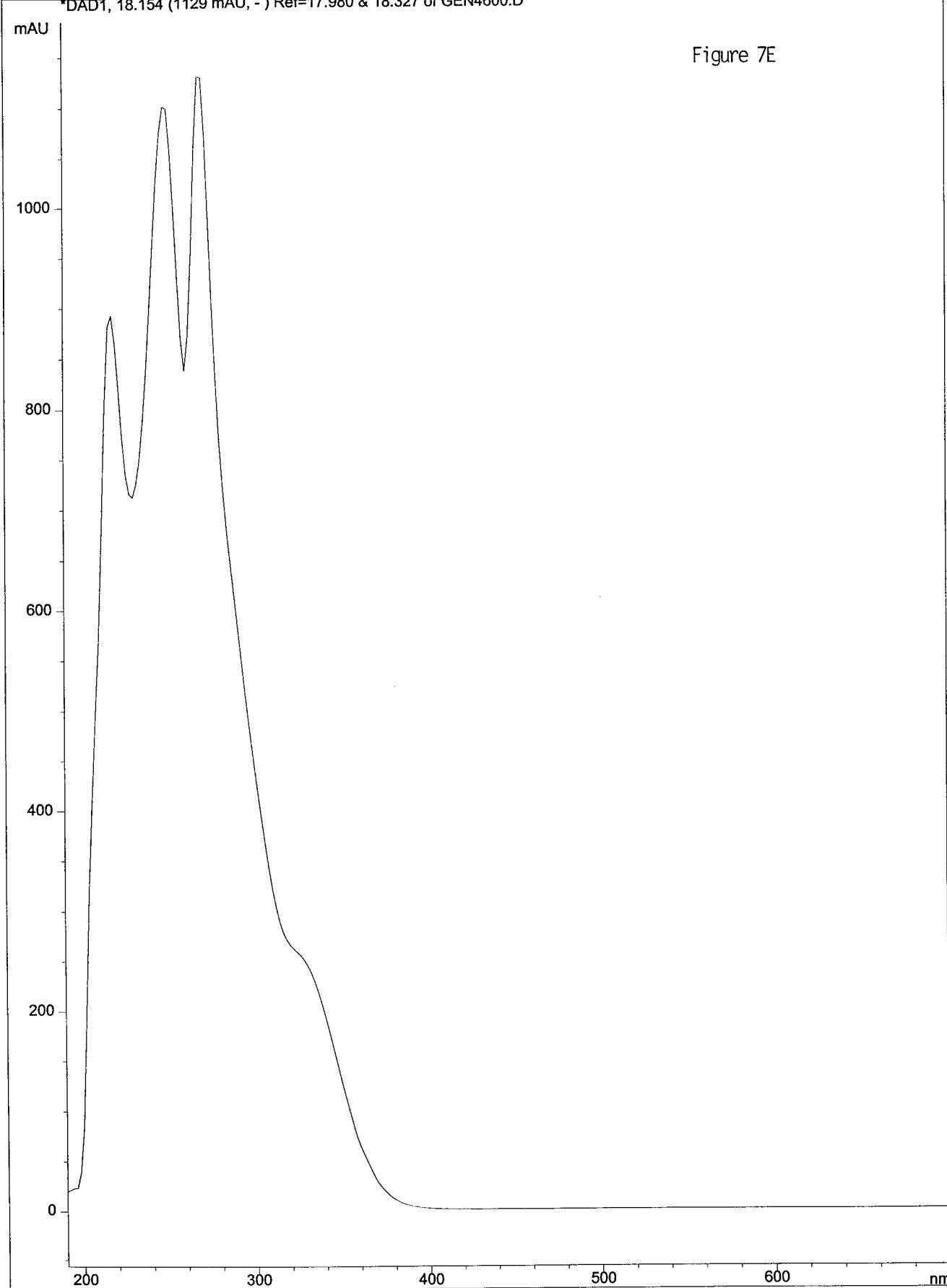


Figure 7E

EGF/ANB/GEN (1:10,1:10) 1 HRLWUV 4/6/00  
 Lichrospher column (RP-18, 5 um) Flow = 1 ml/min

=====
 =====  
 Injection Date : 4/6/00 3:02:10 PM Seq. Line : 5  
 Sample Name : E/A/G 1:10, 1:10 Vial : 5  
 Acq. Operator : Joe Inj : 1  
 Inj Volume : 100  $\mu$ l

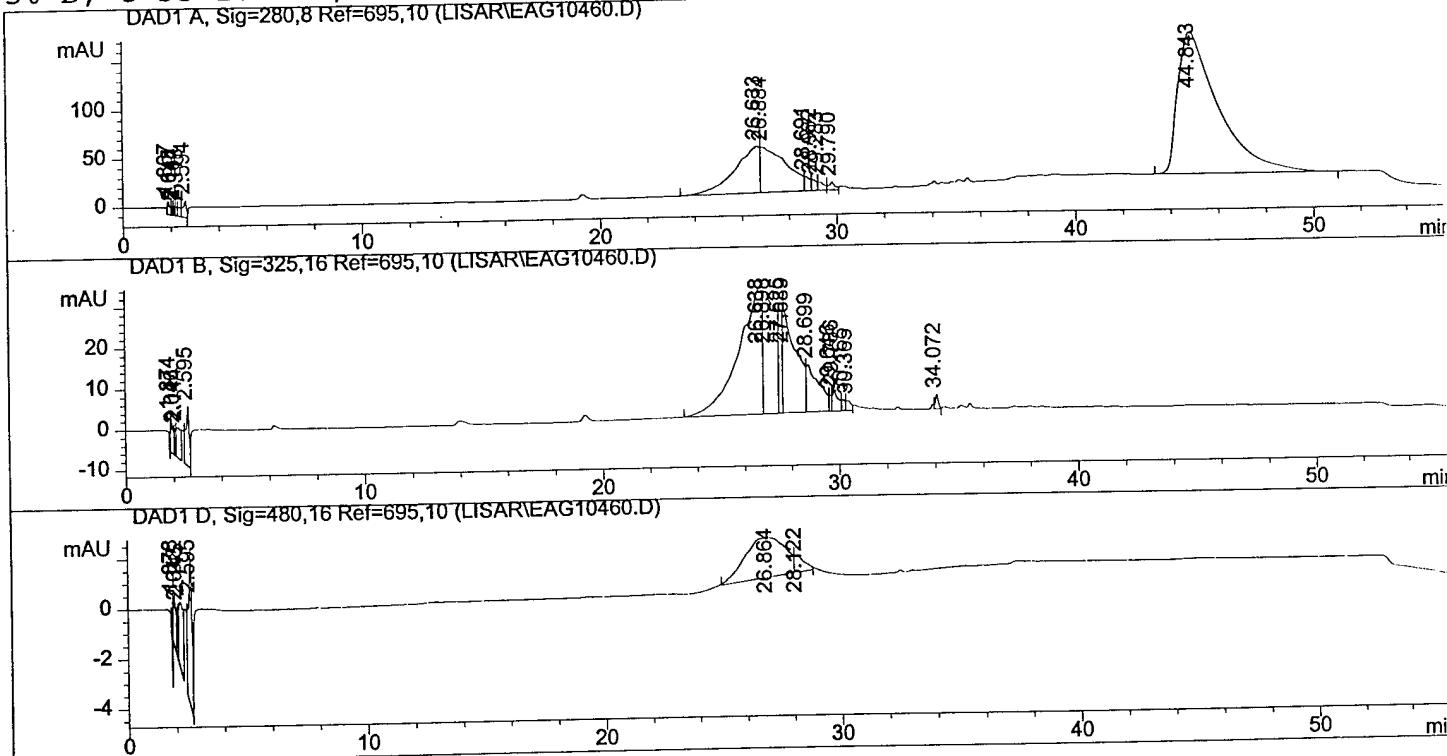
Different Inj Volume from Sequence ! Actual Inj Volume : 25  $\mu$ l

Sequence File : C:\HPCHEM\1\SEQUENCE\EGF4600.S

Method : C:\HPCHEM\1\METHODS\LISAEGF2.M

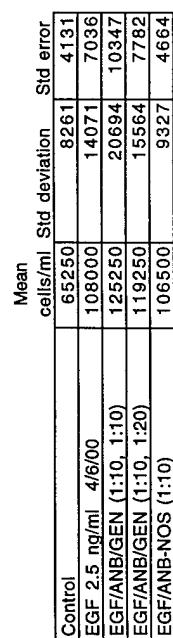
Last changed : 4/6/00 10:26:29 AM by Joe

EGF samples with Lichrospher 100 column. C: H2O, 0.1% TFA D: 80% ACN 20%  
 H2O, 0.1% TFA. Gradient elution: t=0 20% D; t=5 30% D; t=9 38% D; t=20 43%.  
 5% D; t=35 100% D; t=50 100% D; t=55 20% D; t=56 stop.



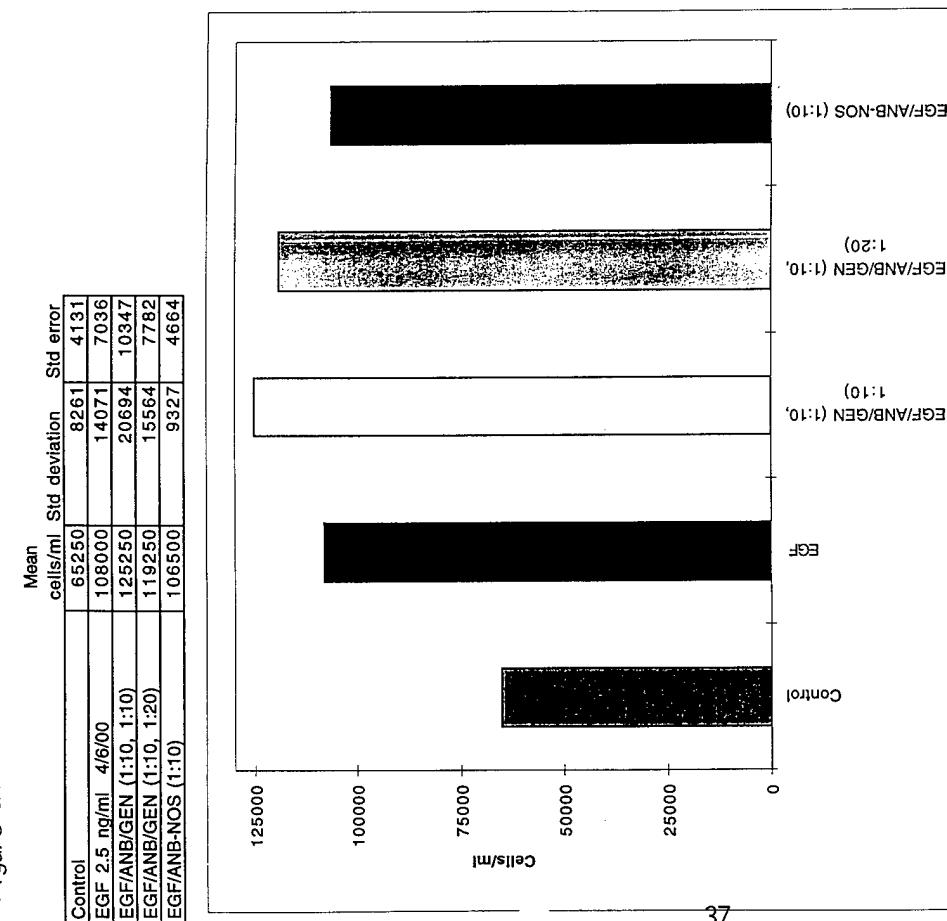
**Figure 7F** - Figure 7F shows the HPLC pattern of EGF-ANB-NOS-Gen made with a 10:1 molar ratio of Genistein to EGF. There is almost no unreacted Genistein in this preparation.

Figure 8A



CRL-1634 cells were incubated for 5 days prior to counting.

Figure 8B



MDAMB231 cells were incubated for 5 days prior to counting.

**Figure 8A and 8B -** Figure 8A shows the growth of CRL-1634 cells in the presence of the EGF-ANB-NOS/EGF-ANB-NOS-Gen conjugates. Figure 8B shows a similar experiment using MDA-MB-231 breast cancer cells.

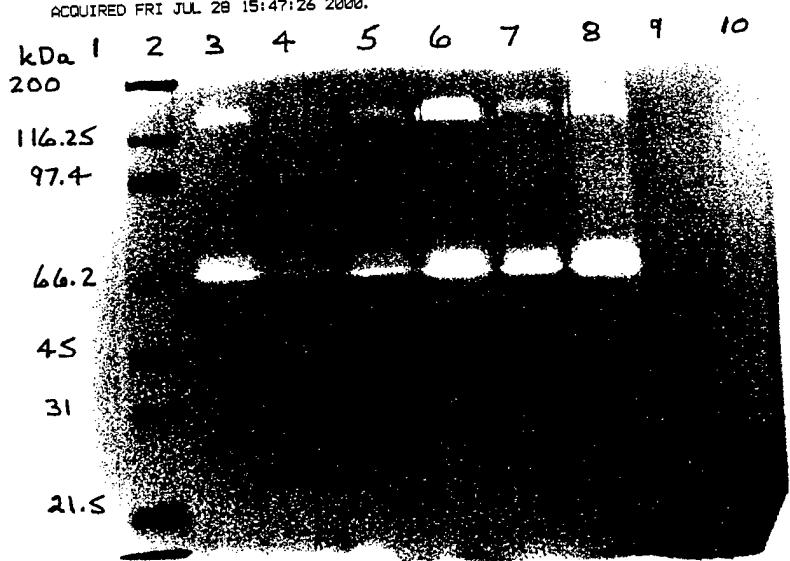
SDS-PAGE  
Zymography

Date: 7-26-00 A

Gel Type: 10% Acrylamide + 0.1% Gelatin

Lane	Description	Amount Loaded
1		
2	MW Standard, broad range, prestained	5 $\mu$ l
3	MDA-MB231 96hrs A Control	10.9
4	Genistein 100 $\mu$ M	10.8
5	DMSO	12.1
6	EGF 10 $\mu$ g/ml	11.7
7	EGF/SAN/Gen 1:10, 1:10 7-17-00	10.8
8	EGF/ANB/Gen 1:10, 1:10 7-17-00	10.6
9		
10		

STRATAGENE EAGLE EYE II 07/26/00 15:47:28  
 Zymography 7/26/00 A  
 IMAGE SIZE (640 x 480 x 8).  
 REAL-TIME ACQUIRE.  
 ACQUIRED FRI JUL 28 15:47:26 2000.



**Figure 9A** - Figure 9A shows a zymography gel of MDA-MB-231 cells incubated with Genistein, DMSO, EGF, and EGF-Gen conjugates. Twenty-five ug of protein were loaded per lane. The daily Genistein concentration was 100 uM(in 1% DMSO) and EGF and the EGF conjugates were at 10 ug/mL. Supernatants were harvested after 96 hr incubation at 37° C.

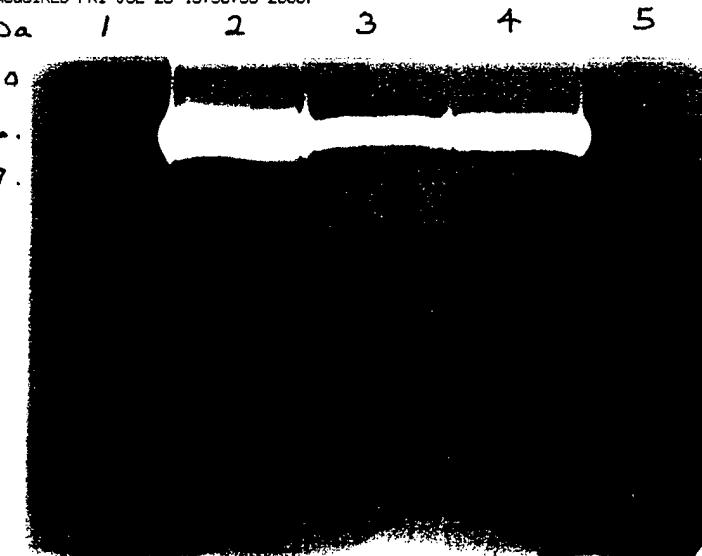
SDS-PAGE  
Zymograph

Date: 7-26-00 C

Gel Type: 10% Acrylamide + 0.1% gelatin

Lane	Description	Amount Loaded
1	MW Standard, broad range, prestained	5 $\mu$ l
2	BT-20 72 hrs Control	32.7
3	↓	36.6
4	↓ EGF/IAuB/GEN	33.8
5		
6		
7		
8		
9		
10	200 116. 97.	

STRATAGENE EAGLE EYE II 07/28/00 15:51:30  
Zymography 7/26/00 C  
IMAGE SIZE (640 x 480 x 8).  
REAL-TIME ACQURE.  
ACQUIRED FRI JUL 28 15:50:59 2000.



**Figure 9B** - Figure 9B shows a zymography gel of BT-20 cells incubated with the EGF-Gen conjugates. A five-well comb was used for this gel and 25  $\mu$ g of protein loaded per lane. Cells were incubated with 10  $\mu$ g/mL daily concentrations of the conjugates and harvested after 72 hr.

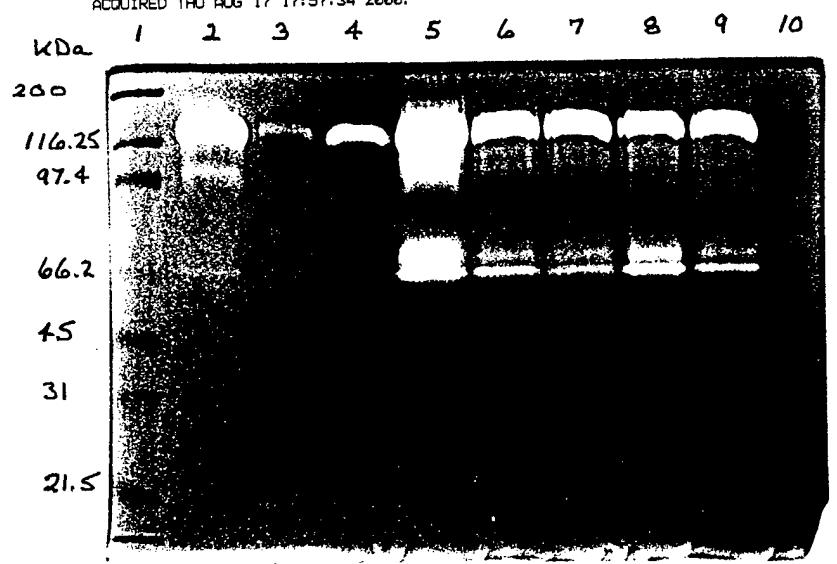
SDS-PAGE  
Zymography

Date: 8-15-00 B

Gel Type: 10% Acrylamide, 0.1% gelatin

Lane	Description	Amount Loaded
1	MW Standard, broad range, prestained	5 $\mu$ l
2	SQ-20B 72 hours 8-8-00 Control	9.8
3		Genistein 100 $\mu$ M
4		DMSO 1%
5		EGF 8-7-00 10 $\mu$ g/lne
6		EGF/SAN/GEN 8-7-00 10 $\mu$ g/lne
7		EGF/AWB/GEN 8-7-00 10 $\mu$ g/lne
8		EGF/SAN/GEN 8-7-00 25 $\mu$ g/lne
9		EGF/AWB/GEN 8-7-00 25 $\mu$ g/lne
10		

STRATAGENE EAGLE EYE II 08/17/00 17:57:42  
 Zymography 8/15/00 B  
 IMAGE SIZE (640 x 480 x 8).  
 REAL-TIME ACQUIRE.  
 ACQUIRED THU AUG 17 17:57:34 2000.



**Figure 9C** - Figure 9C shows a zymography gel of SQ-20B cells incubated with Genistein, DMSO, EGF, and two concentrations of the EGF-Gen conjugates. Ten ug of protein were loaded per lane.